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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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THE BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

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I. TRUSTEES

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H. B. Steinbach, University of Minnesota

TO SERVE UNTIL 1958

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T. H. Bullock, University of California, Los Angeles
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Albert I. Lansing, Emory University
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S. Meryl Rose, University of Illinois
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TO SERVE UNTIL 1957

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MARINE BIOLOGICAL LABORATORY
WOODS HOLE
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MASS.

II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I submit herewith the report of the sixty-ninth session of the Marine Biological Laboratory.

Since the close of World War II there have been certain developments at the Laboratory which have reduced the amount of research space available to summer investigators. It has been necessary to set aside some space formerly assigned to investigators for special instrumentation. Seven laboratories are now used for radiobiology, electron microscopy, dehumidified laboratories for special equipment and the personnel responsible for these services. Eight laboratories are used by investigators working on a year-round basis so are not available for the summer investigators. Thus space for about thirty summer investigators has been diverted to other uses.

Every effort has been made to accommodate as many summer investigators as possible which has probably produced serious crowding in some areas. The establishment of the Neuromuscular Training Program will further reduce the research space available for summer investigators. In recent years there has been a marked increase in the number of applicants for research space with many well qualified individuals being turned away every year. Serious consideration should be given to the expansion of our research facilities. At least the Laboratory should pick up the space lost to other activities. This can best be done by replacing the Rockefeller, Botany and Old Lecture Hall with a modern brick building. Additional housing should be constructed at the same time to avoid over-taxing our present housing and dining hall facilities.

1. Grants, Contracts and Contributions

The total income from these sources of support amounted to $204,034.00 in 1956. This represents 40% of the total income of the Laboratory and consists of the following accounts:

American Cancer Soc.—C26AS—Function of Nuclei and Nucleic Acids	$ 18,705.00
American Cancer Soc.—R-7F—Fundamental Studies in Radiobiology	6,600.00
A.E.C.—At 30-1-1343—Program of Research on the Physiology of Marine Organisms Using Radioisotopes	8,450.00
N.I.H.—B643C—Encephalization in Embryonic Development	2,012.00
N.I.H.—SA43PH 423—Investigations of the Microscopic Physiology of Various Forms of Living Marine Life	1,350.00
N.I.H.—B799—Electrical and Mechanical Changes in Muscle	920.00
N.I.H.—RG4359—Biological Research on the Morphology, Ecology, Physiology, Biochemistry and Biophysics of Marine Organisms	40,000.00
N.I.H.—RG-E-4513—Radiation-Induced Paralysis in Protozoa	14,950.00
National Science Found.—G2142—Funds for Biological Research	25,000.00
National Science Found.—G1807—Mechano-Chemical Coupling in Muscle	11,500.00
National Science Found.—G1395—Osmoregulation of Excretion in Tunicates	2,917.00
National Science Found.—G2655—Structure and Function of Proteins	7,750.00
O.N.R.—1497—Studies in Marine Biology	15,000.00
O.N.R.—09701—Studies on Isolated Nerve Fibers	6,450.00
O.N.R.—09702—Investigation of Environmental Factors Influencing Certain Marine Biological Populations in the Woods Hole area	5,000.00
American Philosophical Society	2,500.00
M. B. L. Associates	5,255.00
Eli Lilly Company	5,000.00
Rockefeller Foundation	20,000.00
Upjohn Company	2,000.00
Ciba Company	1,000.00
Grass Trust	1,000.00
Other	675.00
	$204,034.00

2. *Neuromuscular Training Program*

A neuromuscular training program has been set up under the supervision of Dr. Stephen Kuffler, which will have a staff of three investigators and eight postdoctoral Fellows. This is a multidisciplinary program which will be housed in special quarters developed in the Crane Building. The program is being supported by the Public Health Service.

3. *Plant Improvements*

During the past winter the Supply Department Building was completely reconstructed. Extensive changes were made in the internal arrangement of the building which adapt it much more satisfactorily to its various functions. Included in the building will be a study museum containing specimens of the various forms available in the Woods Hole area for research. Mr. Milton B. Gray, who is very well acquainted with the local fauna, will serve as Curator of the Museum.

The Crane Wing of the Brick Building was built in 1913. It has been in serious need of rehabilitation for the past several years. Many of the facilities were not adequate for much of the research currently in progress at the Laboratory. A request for a grant of $415,000 was made to the National Science Foundation to reconstruct and modernize this building. Early in this year, favorable action was taken by the National Science Foundation on this grant request. This reconstruction work will be undertaken between the 1957–1958 summer seasons. The resulting building should be adequate for any type of research undertaken at the Laboratory.

4. Instruction

In line with the Laboratory's policy Dr. Bostwick H. Ketchum will retire as head of the Marine Ecology course, having served a five-year term. He will be succeeded by Dr. Eugene P. Odum of the University of Georgia.

Dr. Stephen Kuffler has resigned as head of the Physiology course to take over the leadership of the Neuromuscular Training Program. He will be succeeded by Dr. W. D. McElroy of the Johns Hopkins University.

The course in Marine Ecology was originally established under Dr. Ketchum's direction who can take real pride in the way it has developed. Dr. Kuffler maintained the course in Physiology at the high level of effectiveness which has characterized it for many years.

5. Retirements

The Laboratory is losing the services of three of its permanent staff who have served the Laboratory most faithfully for many years. Mr. James McInnis is retiring as Manager of the Supply Department, having served in that department for thirty-eight years.

Miss Polly Crowell has been connected with the Laboratory administration for forty-one years and Miss Ruth Crowell with the Supply Department for thirty-seven years. The success of the Laboratory depends in a large measure on the effectiveness of its staff, of which they have been outstanding members.

6. Deaths

This past year the Corporation of the Marine Biological Laboratory suffered the loss of one of its most eminent and loyal members, in the death of Dr. B. M. Duggar. A memorial to Dr. Duggar will be presented at the Annual Meeting of 1957.

At the Annual Meeting of 1956 Dr. Mary Sears read a memorial for Mrs. Priscilla B. Montgomery. Mrs. Montgomery was for many years the Librarian of the Laboratory and over the years her efforts on behalf of the Library have made it what it is today. Her name will always be remembered with affection and pride by all who knew her.

Respectfully submitted,

PHILIP B. ARMSTRONG,
Director

MEMORIAL

Priscilla Braislin Montgomery

by

Mary Sears

28 December 1874–9 August 1956

In 1897, recent graduates of women's colleges came to Woods Hole for summer courses just as they do today. Priscilla Braislin of Crosswicks, New Jersey, arrived from Vassar and enrolled in the Embryology Course. The next year, Thomas Harrison Montgomery came as an investigator and in 1900 he became an instructor in the bird section of the nature study course. Priscilla Braislin was back to take this course after three years of teaching school; the first at Howard Seminary in West Bridgewater and the next two at the Pratt Institute High School in Brooklyn. Following this summer, they became engaged and were married in Crosswicks on September 19, 1901. Within a few years, three sons were born, Thomas Roger in Philadelphia on July 28, 1902, Hugh in Austin, Texas, on April 17, 1904 and Raymond Braislin in Philadelphia on May 5, 1910. In 1908, the young couple purchased the house on Buzzards Bay Avenue now owned by Norman T. Allen. No one could have foreseen then how closely the family was to become associated with the Woods Hole scientific community!

Professor Montgomery died of pneumonia on March 19, 1912, and his widow had to sell the Woods Hole property and go to work to support her family. At the University of Pennsylvania, Mrs. Montgomery worked as an assistant to the editor of the Journal of Biological Chemistry, Professor A. N. Richards. In 1915, she had a fellowship in Dr. McClung's laboratory to enable her to devote more time to work toward an advanced degree—work she was never able to complete. She next taught at Vassar for a year and then became an assistant professor at the University of Maine for the academic year 1918–1919. While there she taught vertebrate anatomy, histology and embryology, as well as undertaking some original studies of her own on the embryology of the chick.

Coming from a family of teachers, one might have supposed that Mrs. Montgomery would have continued in the family tradition, yet she had a drive and a lack of patience which made her temperamentally unsuited for such a career. Thus, in the fall of 1919, she became the "resident assistant librarian in immediate charge of the library" at the Laboratory, and in 1925, she was made Librarian.

It was due mainly to Mrs. Montgomery's respect for, and understanding of, research that the Library grew to its present stature. When she began in 1919 the entire Library had an annual budget of $2000 and was housed in Room 217 of the Crane Building. However, Mrs. Montgomery felt that she was assisting the work of the laboratory in a very real way and consequently her ambition for the Library knew no bounds. With persistence and the continuing help and interest of Dr. Frank R. Lillie and others, she accumulated an enviable collection, today consisting of some 75,000 volumes.

Although not a trained librarian, she knew the scientists' needs and developed an original, yet practical, system for filing journals, and at the same time expanded the reprint collection for circulation so that the journals themselves could always be available in the Library. On Mrs. Montgomery's retirement at the end of 1947, she had achieved her goal for building an outstanding library, which will long stand as a fitting tribute to her memory.

The building of her home on Whitman Road in 1923 marked the end of the years of her great personal struggle. By then her sons' education as a future business man, a doctor and a meteorologist-oceanographer were nearly completed. From then on she had time for sociability as well as work and she will be remembered for her genuine hospitality by many of the summer visitors at the Laboratory from this country and abroad.

1. The Staff, 1956

Philip B. Armstrong, Director, State University of New York, School of Medicine, Syracuse

Senior Staff of Investigation

A. P. Mathews, Professor of Biochemistry, *Emeritus,* University of Cincinnati

ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University
Libbie H. Hyman, American Museum of Natural History
A. C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

Theodore H. Bullock, Associate Professor of Zoology, University of California, Los Angeles, in charge of course
John H. Lochhead, Professor of Zoology, University of Vermont
Norman A. Meinkoth, Associate Professor of Zoology, Swarthmore College
Grover Stephens, Assistant Professor of Zoology, University of Minnesota
John M. Anderson, Associate Professor of Zoology, Cornell University
Howard A. Schneiderman, Assistant Professor of Zoology, Cornell University
Martin W. Johnson, Professor of Marine Biology, Scripps Inst. of Oceanography, University of California, La Jolla
Morris Rockstein, Department of Physiology, New York University, Bellevue Medical Center

III. Laboratory Assistants

Charles H. Baxter, University of California, Los Angeles
Kent Christensen, Harvard University

EMBRYOLOGY

I. Instructors

Mac V. Edds, Jr., Associate Professor of Biology, Brown University, in charge of course
Paul B. Weisz, Associate Professor of Biology, Brown University
Nelson T. Spratt, Jr., Professor of Zoology, University of Minnesota
J. P. Trinkaus, Assistant Professor of Zoology, Yale University
Edgar Zwilling, Associate Professor of Genetics, University of Connecticut

II. Laboratory Assistant

Robert G. Beard, Indiana University

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Gyorgyi, Director, Institute for Muscle Research, Woods Hole
E. S. Guzman Barron, Associate Professor of Biochemistry, University of Chicago

II. Instructors

Stephen W. Kuffler, Associate Professor of Ophthalmology, Wilmer Institute, Johns Hopkins Medical School, in charge of course
Francis D. Carlson, Assistant Professor of Biophysics, Johns Hopkins University
Bernard D. Davis, Professor of Pharmacology, New York University, College of Medicine
Erik Zeuthen, Lecturer, Laboratory of Zoophysiology, University of Copenhagen
Raymond E. Zirkle, Professor of Radiobiology, University of Chicago
Herman M. Kalckar, National Institutes of Health
Max A. Lauffer, Professor and Head of Dept. of Biophysics, University of Pittsburgh
Andrew Szent-Gyorgyi, Independent Investigator, The Institute for Muscle Research

III. Laboratory Assistant

Paul Bernstein, Department of Zoology, Columbia University

BOTANY

I. Consultant

Wm. Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Harold C. Bold, Professor of Biology, Vanderbilt University, in charge of course
Robert W. Krauss, University of Maryland
Paul C. Silva, Assistant Professor of Botany, University of Illinois

III. Laboratory Assistant

Raymond A. Galloway, University of Maryland

IV. Collector

Gina Arce, Vanderbilt University

MARINE ECOLOGY

I. Consultant

Alfred C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

Bostwick H. Ketchum, Marine Microbiologist, Woods Hole Oceanographic Institution, in charge of course
Edwin T. Moul, Assistant Professor of Botany, Rutgers University
Charles Jenner, Associate Professor of Zoology, University of North Carolina

III. Assistant

Rudolf Scheltema, George Washington University

THE LABORATORY STAFF, 1956

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian
Carl Schweidenbach, Manager of Supply Department
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

Irvine L. Broadbent
Polly L. Crowell
Mrs. Lila Myers
Nancy Shave
Georgiana Marks

LIBRARY

Mary E. Castellano, Assistant Librarian
Mary A. Rohan
Naomi Botelho
Albert Neal

MAINTENANCE OF BUILDINGS AND GROUNDS

Robert Adams
Edmond Botelho
Arthur Callahan
Robert Gunning
John Head
George A. Kahler
Donald B. Lehy
Alton J. Pierce
James S. Thayer

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
John P. Harlow
Seaver Harlow
Patricia Philpott

SUPPLY DEPARTMENT

Ruth S. Crowell
Milton B. Gray
Walter E. Kahler
Robert Perry
Patricia M. Conway
Geoffrey Lehy
Robert O. Lehy
James McInnis
Bruno Trapasso
H. S. Wagstaff

2. INVESTIGATORS, LALOR AND LILLIE FELLOWS, AND STUDENTS

Independent Investigators, 1956

ABBOTT, ROBINSON S., Assistant Professor of Botany, Cornell University
ADELSON, LIONEL M., Research Associate, National Agricultural College
ALLEN, ROBERT DAY, Assistant Professor of Zoology, University of Michigan
ALSCHER, RUTH P., Associate Professor, Manhattanville College
AMBERSON, WILLIAM R., Professor of Physiology, University of Maryland School of Medicine
ANDERSON, JOHN MAXWELL, Associate Professor of Zoology, Cornell University
ARMSTRONG, PHILIP B., Professor of Anatomy, State Univ. of New York, College of Medicine
ARNOLD, WILLIAM A., Scientific Investigator, Oak Ridge National Laboratory
BANG, FREDERICK B., Professor of Pathobiology, Johns Hopkins University School of Medicine
BENNETT, MIRIAM F., Instructor of Biology, Sweet Briar College
BENESCH, REINHOLD, Marine Biological Laboratory, Woods Hole, Massachusetts
BERGER, CHARLES A., Chairman, Department of Biology, Fordham University
BLUM, HAROLD F., Physiologist, Princeton University
BOETTIGER, EDWARD G., Associate Professor, University of Connecticut
BOLD, HAROLD C., Vanderbilt University
BRADY, ROSCOE O., National Institute of Neurological Diseases and Blindness
BRIDGMAN, ANNA J., Professor of Biology, Agnes Scott College
BROWN, FRANK A., JR., Chairman, Dept. of Biological Sciences, Northwestern University
BRYANT, S. H., Instructor of Pharmacology, University of Cincinnati, College of Medicine
BULLOCK, THEODORE H., Professor of Zoology, University of California at Los Angeles
BUTLER, ELMER G., Professor of Zoology, Princeton University
CAMPBELL, MILDRED A., Instructor of Zoology, Smith College
CARLSON, FRANCIS D., Assistant Professor of Biophysics, Johns Hopkins University
CHAET, ALFRED B., Instructor in Zoology, University of Maine
CHANG, JOSEPH J., Instructor, Department of Biology, Brown University
CHASE, AURIN M., Associate Professor of Biology, Princeton University
CHENEY, RALPH HOLT, Professor of Biology, Brooklyn College
CLAFF, C. LLOYD, Research Associate in Surgery, Harvard Medical School
CLARK, ELLIOT R., Professor *Emeritus* of Anatomy, University of Pennsylvania
CLEMENT, A. C., Associate Professor of Biology, Emory University
CLOWES, G. H. A., Research Director *Emeritus,* Lilly Research Laboratories
COHEN, MELVIN J., Instructor in Biology, Harvard University
COHEN, SEYMOUR S., Professor of Biochemistry, University of Pennsylvania
COLE, KENNETH S., Chief, Laboratory of Biophysics, National Institutes of Health
COLWIN, ARTHUR L., Associate Professor and Lecturer, Queens College
COLWIN, LAURA H., Queens College
COOPERSTEIN, SHERWIN J., Associate Professor of Anatomy, Western Reserve University Medical School
COSTELLO, DONALD P., Kenan Professor of Zoology and chairman of the Department, University of North Carolina
COWGILL, ROBERT W., Instructor in Biochemistry, University of California, Berkeley
CROWELL, SEARS, Assistant Professor of Zoology, Indiana University
CSAPO, A., Rockefeller Institute for Medical Research
DAN, JEAN CLARK, Misaki Marine Biological Station, Japan
DAVIS, BERNARD D., Professor and Chairman of Pharmacology, New York University College of Medicine
DODGE, FREDERICK A., JR., Graduate Fellow, Rockefeller Institute for Medical Research
DWYER, JOHN D., Director, Department of Biology, Saint Louis University
EDDS, MAC V., JR., Associate Professor of Biology, Brown University
EISEN, HERMAN N., Professor of Medicine, Washington University School of Medicine
ELLIOTT, ALFRED M., Professor of Zoology, University of Michigan
FITZHUGH, RICHARD, Biophysicist, National Institutes of Health
FREYGANG, WALTER H., S. A. Surg. (R), U. S. Public Health Service

Gosselin, Robert E., Assistant Professor of Pharmacology, University of Rochester, School of Medicine
Green, James W., Associate Professor of Physiology, Rutgers University
Green, Maurice, Instructor of Biochemistry, University of Pennsylvania
Green, Paul B., Junior Fellow, Harvard University
Greif, Roger L., Associate Professor of Physiology, Cornell University Medical College
Grosch, Daniel S., Associate Professor of Genetics, North Carolina State College
Grundfest, Harry, Associate Professor of Neurology, College of Physicians and Surgeons
Guttman, Rita, Assistant Professor of Biology, Brooklyn College
Hagerman, Dwain D., Research Fellow, Harvard Medical School
Hagiwara, S., Visiting Scientist, National Institutes of Health
Harvey, Ethel Browne, Research in Biology, Princeton University
Harvey, E. Newton, Professor of Physiology, Princeton University
Hayashi, Teru, Associate Professor of Zoology, Columbia University
Haywood, Charlotte, Professor of Physiology, Mount Holyoke College
Heilbrunn, L. V., Professor of Zoology, University of Pennsylvania
Henley, Catherine, Research Associate, University of North Carolina
Hervey, John P., Electronic Engineer, Rockefeller Institute for Medical Research
Holz, George G., Jr., Assistant Professor of Zoology, Syracuse University
Holtzer, Howard, Assistant Professor of Anatomy, University of Pennsylvania Medical School
Howard, Robert S., Assistant Professor of Biological Sciences, University of Delaware
Hunter, F. R., Assistant Professor, Roosevelt University
Hyde, Beal B., Assistant Professor of Plant Sciences, University of Oklahoma
Jacobs, William P., Associate Professor, Princeton University
Jenkins, George B., Professor *Emeritus* of Anatomy, George Washington University
Jenner, Charles E., Associate Professor of Zoology, University of North Carolina
Johnson, Frank H., Associate Professor of Biology, Princeton University
Johnson, Martin W., Professor of Marine Biology, Scripps Institution of Oceanography
Kalckar, Herman M., Visiting Scientist, National Institutes of Health
Karush, Fred, Associate Professor of Immunology, The Children's Hospital of Philadelphia
Kao, C. Y., Rockefeller Institute for Medical Research
Karlson, Peter, Max-Planck Institüt für Biochemi, Tübingen, Western Germany
Kemp, Norman E., Assistant Professor of Zoology, University of Michigan
Kempton, Rudolf T., Professor and Chairman, Department of Zoology, Vassar College
Keosian, John, Professor of Biology, Rutgers University
Kind, C. Albert, Assistant Professor of Biochemistry, University of Connecticut
Kleinholz, L. H., Professor of Biology, Reed College
Klotz, Irving M., Professor of Chemistry and Biology, Northwestern University
Kostellow, Adele B., Damon Runyon Fellow, Albert Einstein College of Medicine
Krahl, Maurice E., Professor of Physiology, University of Chicago
Lansing, Albert I., Professor of Anatomy, Emory University
Lauffer, Max A., Professor and Head of Dept. of Biophysics, University of Pittsburgh
Lavoie, Marcel E., Instructor in Zoology, University of New Hampshire
Lazarow, Arnold, Professor and Head of Dept. of Anatomy, University of Minnesota
Lees, A. D., Principal Scientific Officer, Agricultural Research Council Unit of Insect Physiology, England
Lehmann, Fritz E., Professor of Zoology, University of Berne, Switzerland
Levine, Lawrence, Instructor of Zoology, Wayne University
Lewin, Ralph A., Grantee, National Institutes of Health
Lochhead, John H., Professor of Zoology, University of Vermont
Lorand, Laszlo, Assistant Professor of Chemistry, Northwestern University
Lovelace, Roberta, Assistant Professor of Biology, University of South Carolina
Lubin, Martin, Associate in Pharmacology, Harvard Medical School
Lumb, Ethel Sue, Assistant Professor of Zoology, Vassar College
Lynch, William F., Professor of Biology, St. Ambrose College
Maas, Werner K., Assistant Professor of Pharmacology, New York University College of Medicine
Marshak, Alfred, Marine Biological Laboratory, Woods Hole, Massachusetts

Marsland, Douglas, Professor of Biology, Washington Square College, New York University
Meinkoth, Norman A., Associate Professor of Biology, Swarthmore College
Menkin, Valy, Head of Experimental Pathology, Agnes Barr Chase Foundation, Temple University
Metz, Charles B., Associate Professor of Zoology, Florida State University
Monroy, Alberto, Professor of Comparative Anatomy, University of Palermo, Italy
Moore, John W., Associate Chief, Laboratory of Biophysics, National Institutes of Health
Mullins, L. J., Associate Professor of Biophysics, Purdue University
Musacchia, X. J., Associate Professor of Biology, St. Louis University
Nace, Paul Foley, Associate Professor of Anatomy, New York Medical College
Needler, Mary E., Graduate Student and Demonstrator, University of Toronto
Osterhout, W. J. V., Rockefeller Institute for Medical Research
Parker, Johnson, Assistant Professor of Botany, University of Idaho
Parpart, Arthur K., Professor and Chairman, Department of Biology, Princeton University
Paterson, Mabel, Instructor in Zoology, Vassar College
Person, Philip, Chief, Dental Research, V. A. Hospital
Pierce, Madelene E., Professor of Zoology, Vassar College
Plough, Harold H., Professor of Biology, Amherst College
Proctor, Nathaniel K., Professor of Biology, Morgan State College
Prosser, C. Ladd, Professor of Physiology, University of Illinois
Ratliff, Floyd, Rockefeller Institute for Medical Research
Ray, Charles, Assistant Professor of Biology, Emory University
Rebhun, Lionel I., Instructor in Anatomy, University of Illinois
Reed, Clark P., School of Hygiene, Johns Hopkins University
Rockstein, Morris, Associate Professor of Physiology, New York University College of Medicine
Rogers, K. T., Assistant Professor of Zoology, Oberlin College
Rosenthal, Theodore B., Associate Professor of Anatomy, Emory University
Rossi, Harold H., Associate Professor of Radiology, College of Physicians and Surgeons
Roth, Jay S., Associate Professor of Biochemistry, Hahnemann Medical College
Rugh, Roberts, Associate Professor of Radiology, Columbia University
Saroff, H. A., Scientist, USPHS, National Institutes of Health
Scharrer, Ernst A., Professor and Chairman of Dept. of Anatomy, Albert Einstein College of Medicine
Schechter, Victor, Associate Professor of Biology, City College of New York
Schneiderman, Howard A., Assistant Professor of Zoology, Cornell University
Schuh, Joseph E., Assistant Professor of Biology, St. Peter's College
Scott, Dwight B. McNair, Assistant Professor of Biochemistry, University of Pennsylvania
Scott, Sister Florence M., Professor of Biology, Seton Hill College
Scott, George T., Professor of Zoology, Oberlin College
Senft, Alfred W., Falmouth Medical Associates
Shanes, Abraham M., Physiologist, National Institutes of Health
Silva, Paul C., Assistant Professor of Botany, University of Illinois
Slifer, Eleanor H., Associate Professor of Zoology, State University of Iowa
Speidel, Carl C., Professor and Chairman of Anatomy Dept., University of Virginia Medical School
Spratt, Nelson T., Professor of Zoology, University of Minnesota
Spyropoulos, Constantine, National Institutes of Health
Stearns, Richard N., Instructor in Physiology, Albert Einstein College of Medicine
Steele, Richard H., Visiting Investigator, Muscular Dystrophy Associations of America, Inc.
Stefanelli, Alberto, Director, Universita di Rome
Steinberg, Malcolm S., Graduate Student, University of Minnesota
Stephens, Grover C., Assistant Professor of Zoology, University of Minnesota
Stunkard, Horace W., Fishery Research Biologist, New York University
Sturtevant, Alfred H., Professor of Genetics, California Institute of Technology
Szent-Gyorgyi, Albert, Marine Biological Laboratory, Woods Hole, Mass.
Szent-Gyorgyi, Andrew, Research Associate, Marine Biological Laboratory, Woods Hole, Mass.
Tasaki, I., Chief, Section on Special Senses, National Institutes of Health

TAYLOR, WILLIAM RANDOLPH, Professor of Botany, University of Michigan
TOKAY, ELBERT, Associate Professor of Physiology, Vassar College
TRINKAUS, J. P., Assistant Professor of Zoology, Osborn Zoological Laboratory, Yale University
TYLER, DAVID B., Professor of Pharmacology and Director of Department, University of Puerto Rico, School of Medicine
URETZ, ROBERT B., Instructor in Biophysics, University of Chicago
deVILLAFRANCA, GEORGE W., Assistant Professor of Zoology, Smith College
VILLEE, CLAUDE A., Assistant Professor of Biochemistry, Harvard Medical School
VINCENT, W. S., Instructor in Anatomy, Upstate Medical Center, State University of New York
WEBB, H. MARGUERITE, Assistant Professor of Physiology, Goucher College
WEISZ, PAUL B., Associate Professor of Biology, Brown University
WHITING, ANNA R., Lecturer in Zoology, University of Pennsylvania
WHITING, P. W., Professor of Zoology *Emeritus,* University of Pennsylvania
WICHTERMAN, RALPH, Professor of Biology, Temple University
WIERCINSKI, FLOYD J., Assistant Professor of Physiology, Hahnemann Medical College
WILBER, CHARLES G., Chief, Comparative Physiology Branch, Army Chemical Center
WILLEY, C. H., Chairman, Department of Biology, New York University, University College
WILSON, T. G., Research Associate, Princeton University
WILSON, WALTER L., Assistant Professor of Physiology and Biophysics, College of Medicine, University of Vermont
WOOTTON, DONALD M., Woods Hole, Mass.
WITTENBERG, JONATHAN B., Assistant Professor of Biochemistry, Albert Einstein College of Medicine
ZEUTHEN, ERIK, Lecturer, University of Copenhagen
ZIMMERMAN, ARTHUR M., Research Associate, Washington Square College, New York University
ZIRKLE, RAYMOND E., Professor of Radiology, University of Chicago
ZWEIFACH, B. W., Associate Professor of Pathology, New York University College of Medicine
ZWILLING, EDGAR, Associate Professor of Genetics, University of Connecticut

Beginning Investigators, 1956

AMENTA, PETER S., Pre-doctoral Student, University of Chicago
BAXTER, CHARLES H., Teaching Assistant, University of California at Los Angeles
BEARD, ROBERT G., Graduate Student, Indiana University
DAVIDSON, MARGARET E., Demonstrator-Curator in Zoology, McGill University
FRIZ, CARL T., Research Assistant, University of Minnesota
HONEGGER, CAROL M., Graduate Student, University of Pennsylvania
KANE, ROBERT EDWARD, Graduate Student, Johns Hopkins University
KURAHASHI, KIYOSHI, Research Fellow, National Institutes of Health
LARIS, PHILIP C., Graduate Student, Princeton University
LEVIN, CLINTON N., Student, New York University College of Medicine
MOOS, CARL, Research Associate, Northwestern University
MORRILL, JOHN B., Graduate Student, Florida State University
NEWMAN, ANNA E., Research Fellow, Western Reserve University
PUGNO, SANDRA L., Post-doctoral Fellow, Yale University
REIMER, STANLEY M., Graduate Student, Rutgers University
STEVENSON, J. ROSS, Graduate Student, Northwestern University
TAYLOR, ROBERT E., Research Physiologist, National Institutes of Health
TUCKER, MARIE, Graduate Student, University of Illinois
TUNIK, BERNARD D., Graduate Student, Columbia University
WHEAT, ROBERT W., Research Fellow, National Institutes of Health

Research Assistants, 1956

ADAMS, TERRY, 86 Chestnut Street, Boston 8, Massachusetts
ARCE, GINA, Vanderbilt University
BAIRD, SPENCER, Institute for Muscle Research
BARNER, HAZEL, Children's Hospital of Philadelphia

Bernstein, Paul W., New York University College of Medicine
Brown, Robert A., Northwestern University
Cagle, Julien, Princeton University
Cranston, Margaret B., Radcliffe Graduate School
Cullerton, John M., University of Pennsylvania
Davis, Roger E., University of Wisconsin
Drake, John W., California Institute of Technology
Erdman, Howard E., North Carolina State College
Finck, Henry, University of Pennsylvania School of Medicine
Gorman, Donald J., New York University Medical School
Greenlees, Janet, Rutgers University
Harsch, Mary, Rutgers University
Hochgraf, Helen, Smith College
Karreman, George, Research Associate, Institute for Muscle Research
Kogel, Joan, Albert Einstein College of Medicine
Kramer, Alan D., New York University School of Medicine
Kurland, Charles G., Cornell University
Lamb, George A., State University of New York Medical School at Syracuse
Langer, Ira J., State University of New York Medical School at Syracuse
Laurie, John S., Johns Hopkins University
Levine, Lenore S., New York University School of Medicine
Lichtenstein, Janet, Children's Hospital of Philadelphia
Loeb, Timothy, Reed College
McLaughlin, Jane A., Institute for Muscle Research
Matheson, Gail E., Yale University
Middlebrook, W. R., Institute for Muscle Research
Mingioli, Elizabeth S., New York University College of Medicine
Palmer, Roger Farley, Florida State University
Philpott, Delbert E., Research Associate, Institute for Muscle Research
Oberlander, Marcia I., State University of New York College of Medicine at Syracuse
Outka, Darryll E., University of California
Pflueger, Otto H., Reed College
Reich, Melvin, Rutgers University
Rosenthal, Elizabeth, National Institutes of Health
Ross, Samuel M., Brooklyn College
Rowe, Edward C., University of Michigan
Shriner, Joan, Northwestern University
Sie, Edward, Princeton University
Smiley, Sheldon, State University of New York
Smith, Zoe Hollingsworth, North Carolina State College
Sperelakis, Nick, University of Illinois
Strauss, Paul H., New York University College of Medicine
Strickholm, Alfred, University of Minnesota
Szent-Gyorgyi, Eva, Institute for Muscle Research
Szent-Gyorgyi, Marta, Institute for Muscle Research
Vozick, Michael W., Columbia University

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS.

Library Readers, 1956

Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Bentley, Ronald, Assistant Professor of Biochemistry, University of Pittsburgh
Bodansky, Oscar, Chief, Clinical Biochemistry, Sloan-Kettering Institute
Braitenberg, Valentine, Yale Medical School
Chace, John A., Kirksville College of Osteopathy and Surgery
Deane, Helen W., 387 Harvard Street, Cambridge 38, Massachusetts
Dixon, F. J., Jr., Professor and Chairman Department of Pathology, University of Pittsburgh School of Medicine
Dorfman, Albert, Associate Professor of Pediatrics, University of Chicago

Freund, Jules, Chief, Division of Immunology, The Public Health Research Institute of New York
Fries, E. F. B., Associate Professor, The City College of New York
Gabriel, Mordecai L., Associate Professor of Biology, Brooklyn College
Ginsberg, Harold S., Associate Professor of Preventive Medicine, Western Reserve University, School of Medicine
Glass, H. Bentley, Professor of Biology, Johns Hopkins University
Glasser, Richard L., University of Maryland
Goldman, Stanford, Professor of Electrical Engineering, Syracuse University
Goldthwait, David A., Senior Clinical Instructor in Medicine, Western Reserve University
Grant, Philip, Research Associate, Department of Embryology, Institute for Cancer Research
Gudernatsch, Frederick, Cornell University Medical College
Gurewich, Vladimir, Associate Visiting Physician, Bellevue and Metropolitan Hospitals
Herbert, Edward, Instructor in Biology, Massachusetts Institute of Technology
Jacobs, M. H., Professor *Emeritus* of General Physiology, University of Pennsylvania
Kabat, Elvin A., Professor of Microbiology, Columbia University
Kohn, Robert R., Research Fellow, Benjamin Rose Hospital
Lein, Allen, Associate Professor of Physiology, Northwestern University Medical School
Levy, Arthur L., Research Biochemist, St. Vincent's Hospital
Levine, Rachmiel, Chairman, Department of Medicine, Michael Reese Hospital
Ling, Gilbert N., Assistant Professor of Neurophysiology, University of Illinois
Loewi, Otto, Research Professor of Pharmacology, New York University-Bellevue Medical Center
Love, Lois H., Research Associate, National Research Council
McDonald, Sister Elizabeth, Chairman, Department of Biology, College of Mt. St. Joseph
Pick, Joseph, Associate Professor in Anatomy, New York University-Bellevue Medical Center
Rennie, Donald W., Research Fellow, Harvard University School of Medicine
Root, Walter S., Professor of Physiology, College of Physicians and Surgeons
Rose, S. Meryl, Professor of Zoology, University of Illinois
Rubin, Saul H., Director, Pharmaceutical and Biochemical Research, Hoffmann-La Roche
Scott, Allan, Chairman, Department of Biology, Colby College
Scott, Thomas F. M., Research Professor of Pediatrics, Children's Hospital
Stockard, Alfred H., Professor of Zoology, University of Michigan
Sulkin, S. Edward, Professor and Chairman, Department of Microbiology, University of Texas Southwestern Medical School
Swift, Hewson, Associate Professor of Zoology, University of Chicago
Teas, Howard J., Plant Physiologist, Federal Experiment Station, Mayaguez, Puerto Rico
Trurnit, Hans J., Member of the Scientific Staff, R. I. A. S., Baltimore
Tyler, Albert, Professor of Embryology, California Institute of Technology
Veis, Arthur, Research Chemist, Armour and Company
Vishniac, Wolf, Assistant Professor of Microbiology, Yale University
Wainio, Walter W., Associate Professor of Biochemistry, Rutgers University
Waterman, Allyn J., Professor of Biology, Williams College
Warner, Robert C., Associate Professor of Biochemistry, New York University College of Medicine
Wheeler, George E., Instructor in Biology, Brooklyn College
Yntema, Chester L., Professor of Anatomy, State University of New York College of Medicine

Lalor Fellows, 1956

S. H. Bryant
M. Cohen
M. Green
P. Gross
D. Hagerman
P. Karlson
L. Levine
A. Lees
W. Maas
L. Rebhun

Lillie Fellow, 1956

A. Stefanelli

Students, 1956

BOTANY

Adair, Elizabeth J., Yale University
Anderson, Robert G., University of Nebraska
Cornett, Margaret E., Radcliffe College
Dick, Stanley, Brooklyn College
Felitti, Vincent J., Dartmouth College
Forbes, Patricia R., Acadia University
Korn, Robert W., Marquette University
Laurencot, Henry J., Jr., Fordham University
Lind, Elizabeth A., Wellesley College
McLeod, Guy C., Waquoit, Massachusetts
Posey, Janet A., West Virginia University
Smalley, Alfred E., University of Georgia
Spyrides, George J., Dartmouth College
Talamo, Richard Charles, Harvard University
Ward, Vernon U., Dartmouth College
Whittier, Dean Page, University of Massachusetts
Wilbois, Annette D., Indiana University
Willson, Dan L., University of Oklahoma

EMBRYOLOGY

Abbott, Joan, Washington University
Babcock, Richard G., University of Michigan
Brice, Martha C., Oberlin College
Bull, Alice Louise, Wellesley College
Burke, Joseph A., S.J., Fordham University
Chaube, Shakuntala, Osborn Biological Laboratory
Coleman, John R., Indiana University
DeCosta, Mari J., Radcliffe College
Filosa, Michael F., Princeton University
Fulton, Chandler M., Brown University
Glasser, Jay H., University of Connecticut
Hinsch, Gertrude W., Iowa State College
Hollenback, James G., Marquette University
Huver, Charles W., University of Wisconsin
Katoh, Arthur K., University of Illinois
Kinyon, Nancy, Northwestern University
Laufer, Hans, Cornell University
McDowell, James W., Dartmouth College
Outten, Lora M., Cornell University
Paul, Sister Clarence, St. John's University
Raff, Neil C., Amherst College
Ray, Frances L., Columbia University
Robinson, James Alan, Wesleyan University
Rothman, Maxine, Indiana University
Ruggieri, George D., S.J., St. Louis University
Steinmuller, David, Swarthmore College
Tsai, Lie Sha, Yale University
Tweedell, Joan E. Werber, University of Maine

PHYSIOLOGY

Bade, Maria L., Omaha, Nebraska
Bauman, Norman, New York University College of Medicine
Brand, Eugene D., University of Virginia

Campbell, James W., University of Oklahoma
Christiensen, Albert K., Harvard University
Clark, Eloise E., University of North Carolina
Evans, David R., Johns Hopkins University
Goldstein, Judith H., Harvard University
Gonzalez, Maria, University of São Paulo, Brazil
Gordon, Maria F., Cox Institute, Pennsylvania University
Greene, Lewis J., Rockefeller Institute for Medical Research
Gross, Ruth T., Stanford University Medical School
Kim, Soon Won, Columbia University
Kostyo, Jack L., Cornell University
Lenhert, Paul G., Johns Hopkins University
Newman, Anna E., Western Reserve University
Paik, Woon Ki, Dalhousie University
Pugno, Sandra L., Osborn Zoological Laboratory
Ruark, Margaret A., Yale University
Schooley, Caroline N., University of California
Schooley, John C., University of California
Schwartz, James H., New York University College of Medicine
Smith, Geneva A., Mount Holyoke College
Temin, Howard M., California Institute of Technology
Theis, Roger Elliot, Harvard University
Ting, Robert C. Y., Amherst College
Wilt, Fred H., Indiana University
Yamamoto, Robert T., University of Illinois
Young, Robert R., Yale University
Zorzoli, Anita, Vassar College

INVERTEBRATE ZOOLOGY

Andrews, Fred B., Indiana University
Baren, Carl F., Brooklyn College
Bartol, Roberta B., Dunbarton College
Bingham, Eula L., University of Cincinnati
Bishop, Alison, Cornell University
Boohar, Richard K., Drew University
Burger, Charles L., University of Illinois
Chamberlain, Norman A., University of North Carolina
Clark, George A., Amherst College
Collier, Nancy V., Goucher College
Douglas, Donald, Oberlin College
Dowling, Richard A., State University of Iowa
Eiger, Joan V., Harvard University
Ellis, John F., Amherst College
Fiore, Carl, Fordham University
Fleischman, Julian B., Harvard University
Ford, Elizabeth, Washington University
Friedl, Frank Edward, University of Minnesota
Goldberg, Morton F., Harvard College
Goudsmit, Esther M., University of Michigan
Guignon, Ernest F., Washington and Jefferson College
Hammer, Beverly A., Randolph-Macon Woman's College
Hammonds, Joanne, Chatham College
Harmon, Wallace, Syracuse University
Harman, Walter J., University of Illinois
Hendricks, Frederick B., DePauw University
Hichar, Joseph K., Harvard University
Holmes, William F., University of Pennsylvania
Holt, Kathleen, Western Maryland College

Huber, Ivan, University of Maryland
Joline, Laurence T., Washington University
Kanwisher, Joan T., Woods Hole, Mass.
Karakashian, Stephen, Drew University
Kirchen, Robert V., University of Michigan
Koukides, Melpomeni, University of Pennsylvania
Lassen, Ida, Elmira College
London, Abram M., Harvard University
McNab, Brian K., University of Wisconsin
Moffat, Grace H., City College of New York
Morrow, Cynthia J., Tufts College
Plumb, Mary E., Vassar College
Purple, Richard, Hamilton College
Rabinowitch, Victor, University of Illinois
Reppert, Jere Anne, Goucher College
Robertson, Robert, Harvard University
Schaefer, Carl W., II, Oberlin College
Scheuing, Marilyn R., State Medical School
Spock, Michael, Antioch College
Sullivan, Helen M., Marquette University
Taub, Stephen, Indiana University
Taylor, Robert E., University of Delaware
Tefft, Edwin R., Fordham University
Tester, Ruth E., Hunter College
Wharton, Thalia J., Mount Holyoke College
Yesair, David W., Cornell University

ECOLOGY

Fersahl, Sister John Baptist, Fordham University
Foster, Walter S., Colby College
Gates, John O., Cornell University
Gilbert, Ann C., Columbia University
Hirchinson, Vivienne, Mount Holyoke College
Jones, Sarah R., Connecticut College
Strelecki, Raymond F., Drew University
Van Dyk, N. Joanne, University of New Hampshire
Weinstock, Ammon, Brandeis University

3. Fellowships and Scholarships, 1956

Arsenious Boyer Fellowship:
Father Wm. Lynch

Lucretia Crocker Scholarship:
Robert Anderson, Botany Course
N. Joanne Van Dyk, Ecology Course

The Gary N. Calkins Scholarship:
Robert Robertson, Invertebrate Zoology Course

The Edwin Grant Conklin Scholarship:
Hans Laufer, Embryology Course

Emma Coote Drew Scholarship:
Eloise Clark, Physiology Course
Bio Club Scholarship:
Grace Moffatt, Invertebrate Zoology Course

The Edwin Linton Scholarship:
Ernest Guignon, Invertebrate Zoology Course

4. TABULAR VIEW OF ATTENDANCE, 1952–1956

	1952	*1953*	*1954*	*1955*	*1956*
INVESTIGATORS—TOTAL	306	310	208	250	304
Independent	172	176	180	162	184
Under Instruction	38	37	20	9	20
Library Readers	49	46	52	54	50
Research Assistants	47	51	46	25	50
STUDENTS—TOTAL	123	136	134	148	140
Zoology	55	55	56	56	55
Embryology	23	30	29	30	28
Physiology	27	31	28	30	30
Botany	11	11	12	19	18
Ecology	7	9	9	13	9
TOTAL ATTENDANCE	429	446	432	398	444
Less persons represented as both students and investigators	2		5		2
	427	446	427	398	442
INSTITUTIONS REPRESENTED—TOTAL	149	155	136	129	130
By investigators	92	90	104	95	97
By students	57	65	32	34	33
SCHOOLS AND ACADEMIES REPRESENTED					
By investigators	1		2	3	3
By students	3	1	1	2	1
FOREIGN INSTITUTIONS REPRESENTED					
By investigators	7	15	11	8	9
By students	2	6	13	6	6

5. COOPERATING AND SUBSCRIBING INSTITUTIONS, 1956

Cooperating Institutions

Amherst College
American Cancer Society
American Philosophical Society
Brooklyn College
Brown University
Bryn Mawr College
California Institute of Technology
Children's Hospital of Philadelphia
City College of New York
Colby College
College of Mt. St. Joseph on the Ohio
Columbia University
Columbia University, College of Physicians and Surgeons
Cornell University
Cornell University Medical School
Duke University
Elmira College
Emory University
Florida State University
Fordham University
Grass Foundation
Hahnemann Medical College
Harvard University
Harvard University Medical School
Indiana University
Institute for Cancer Research
Institute for Muscle Research
Johns Hopkins University
Johns Hopkins University Medical School
Lalor Foundation
Eli Lilly and Company
Marquette University
Morgan State College
Mount Holyoke College
National Institutes of Health
National Science Foundation
New York University—Heights
New York University, College of Medicine
New York University—Washington Square College
North Carolina State College
Northwestern University
Oberlin College
Office of Naval Research
Princeton University
Public Health Institute of New York
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University

Saint Louis University
Sloan-Kettering Institute
Southwestern Medical College
State University of Iowa
State University of New York, College of Medicine, at Syracuse
Syracuse University
Temple University
Tufts College
University of Chicago
University of Connecticut
University of Illinois
University of Maryland School of Medicine
University of Michigan
University of Minnesota
University of Pennsylvania
University of Pennsylvania Medical School
University of Pittsburgh
University of Rochester
University of Virginia, School of Medicine
University of Wisconsin
Vassar College
Washington University
Washington and Jefferson College
Wellesley College
Wesleyan University
Western Reserve University
Yale University
Yale University Medical School

Subscribing Institutions

Acadia University
Armour and Company
Brandeis University
Chatham College
City College of New York
Drew University
Albert Einstein College of Medicine
Ethicon Corporation
Falmouth Medical Associates
Goucher College
Hamilton College
House of Good Samaritan
Hunter College
Massachusetts Institute of Technology
National Agricultural College
Purdue University
Michael Reese Hospital
Smith College
University of California
University of Florida
University of Maine
University of Massachusetts
University of New Hampshire
University of Oklahoma
University of Puerto Rico
University of Vermont
Veterans Administration Hospital
Washington University School of Medicine

6. EVENING LECTURES, 1956

June 29
Daniel Mazia ...."Processes in cell reproduction"
July 6
Donald R. Griffin ...."Listening in the dark"
July 13
Harry Grundfest ...."The different kinds of electrical responses and their significance to the organism"
July 20
Vincent du Vigneaud ...."The posterior pituitary hormones"
July 27
W. D. McElroy ...."Recent developments in the biochemistry of light emission"
August 3
F. E. Lehmann ...."Cytoplasmic organization and developmental physiology of the egg of Tubifex"
August 10
V. B. Wigglesworth ...."The insect cuticle"
August 17
Alberto Stefanelli ...."The life cycle of neurons"
August 24
Keith R. Porter ...."The submicroscopic morphology of protoplasm"

7. Tuesday Evening Seminars, 1956

July 10

Joan Wolff and Roberts Rugh ......"The relation of gonad hormones to x-irradiation sensitivity in mice"

Paul R. Gross ...."Amphibian yolk: chemistry and ultrastructure"

Sylvan Nass ...."Amphibian yolk: the phosphoprotein phosphatase system"

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Anthony, Mr. Richard A.
Armstrong, Dr. and Mrs. P. B.
Barbour, Mr. Lucius
Bartow, Mr. and Mrs. Clarence
Bartow, Mrs. Francis D.
Bartow, Mr. and Mrs. Philip
Bell, Mrs. Arthur
Benjamin, Mr. Edward
Bradley, Mr. Albert L.
Bradley, Mrs. Charles Crane
Brown, Mrs. Thornton
Burdick, Mr. Charles L.
Burlingame, Mrs. F. A.
Cahoon, Mrs. Samuel
Calkins, Mr. G. Nathan, Jr.
Calkins, Mrs. Gary N.
Calkins, Mr. Samuel
Carleton, Mrs. Winslow
Claff, Mr. and Mrs. C. Lloyd
Clark, Dr. and Mrs. Alfred Hull
Clark, Mrs. Leroy
Clark, Mr. W. Van Alan
Clowes, Mr. Allen W.
Clowes, Mrs. G. H. A.
Clowes, Dr. and Mrs. George, Jr.
Colton, Mr. H. Seymour
Crane, Miss Louise
Crane, Mrs. W. Carey
Crane, Mrs. W. Murray
Crossley, Mr. and Mrs. Archibald M.
Crowell, Mr. Prince S.
Daniels, Mr. and Mrs. F. Harold
Day, Mr. and Mrs. Pomeroy
Draper, Mrs. Mary C.
Dreyer, Mrs. Frank
Elsmith, Mrs. Dorothy
Enders, Mr. Frederick
Ewing, Mr. Frederick
Fay, Mr. and Mrs. Henry H.
Fisher, Mrs. Bruce Crane
Friendship Fund, Inc.
Frost, Mrs. Eugenia
Galtsoff, Mrs. Eugenia
Gifford, Mr. and Mrs. John A.
Gilchrist, Mr. and Mrs. John A.
Gildea, Dr. and Mrs. E. F.
Green, Miss Gladys W.
Hamlen, Mr. J. Monroe
Harrell, Mr. and Mrs. Joel E.
Harrington, Mr. and Mrs. A. W.
Harrington, Mr. Robert D.
Houston, Mr. and Mrs. Howard E.
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Jewett, Mrs. George F.
Keith, Mr. and Mrs. Harold C.
Kidder, Mrs. Henry M.
King, Mr. Franklin
Koller, Mrs. Lewis
Lawrence, Mr. Milford
Lemann, Mrs. Solen B.
Lobb, Mrs. John
Loeb, Dr. Robert F.
McClintic, Mrs. Guthrie
McKeloy, Mr. John
Marvin, Mrs. Walter T.
Mast, Mrs. S. O.
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Packard, Mrs. Laurence B.
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Williston, Miss Emily
Williston, Miss Mary D.
Williston, Prof. Samuel
Wilson, Mrs. Edmund B.
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V. REPORT OF THE LIBRARIAN

In 1956, the number of currently received journals totalled 1575 (48 new). Of these titles, there were 471 (6 new) Marine Biological Laboratory subscriptions; 607 (12 new) exchanges and 182 (15 new) gifts; 81 (9 new) were Woods Hole Oceanographic Institution subscriptions; 184 (4 new) were exchanges and 50 (2 new) were gifts.

The Laboratory purchased 60 books, received 77 complimentary copies (12 from authors and 65 from publishers), and accepted 25 miscellaneous gifts. The Institution purchased 23 titles and received 5 gifts. The total number of new books accessioned amounted to 190.

By purchase the Laboratory completed 14 journal sets and partially completed 13. The Institution completed four sets and partially completed three. Volumes and numbers received by gift and by exchange completed four sets and partially completed 16 sets.

There were 6112 reprints added to the collection, of which 2326 were of current issue.

At the end of the year, the Library contained 66,590 bound volumes and 202,201 reprints.

The Library sent out on inter-library loan 192 volumes and borrowed 74 for the convenience of the investigators.

Dr. E. Newton Harvey's collection of reprints was processed in 1956 and several thousand were added to the Library's collection. The duplicate material was presented to the University of North Carolina Library.

At the close of the year, Dr. Albert I. Lansing was influential in having an accumulation of reprints sent from the University of Pittsburgh. These will be processed in 1957.

Grateful acknowledgment is extended to Drs. Alfred C. Redfield, Henry Stommel, Ethel B. Harvey, Roberts Rugh, P. W. Whiting, Wm. R. Amberson, Dorothy Wrinch, Helen W. Kaan, and to the Tompkins-McCaw Library, Medical College of Virginia, for valuable and useful contributions of books, reprints and old photographs.

During the summer, the Library Committee, fully aware of the demand for an increase in the purchase of books, took action in securing a larger appropriation for this purpose. A Library Advisory Committee was appointed to recommend titles for purchase in 1957. There are 23 persons on this Committee and it is hoped a very substantial increase in book acquisitions will be realized. Extra funds are also expected in 1957 for the binding of back periodical volumes—work that has been neglected in order to keep the current binding up to date. The steady increase in the number of current periodicals now being published has also made it necessary to request an increase in the budget for 1957.

In August, Mrs. Priscilla B. Montgomery, the former Librarian, passed away. It is due to her foresight during the early days of the Library's rapid growth, that the present system, initiated by her, has proved to be an adaptable and an efficient one. The Library is a very fitting memorial to the loyal service and painstaking work displayed by her during the years 1919–1947. (A memorial paper is contained in the Laboratory's report).

Respectfully submitted,

Deborah L. Harlow,
Librarian

VI. REPORT OF THE TREASURER

The combined market values of securities for the General Fund and the Library at December 31, 1956, amounted to $1,472,265.00 as compared with the total of $1,500,773 as of December 31, 1955. The average yield on the securities was 3.76% of market value and 5.55% of book value. The total uninvested principal cash in the above accounts as of December 31, 1956, was $1,551.72. The securities list held in the Endowment Funds appears in the auditor's report.

The pooled securities had a market value at the end of the year of $242,759.00 with uninvested principal cash in the amount of $430.12. The book value of the securities in this account was $223,341.33. The average yield on market value was 3.77% and 4.10% on book value.

The proportionate interest in the Pooled Fund account of the various Funds as of December 31, 1956, is as follows:

Pension Fund	16.680%
General Laboratory Investments	60.619
Other:	
Bio Club Scholarship Fund	1.768
Rev. Arsenious Boyer Scholarship Fund	2.161
Gary N. Calkins Fund	2.024
Allen R. Memhard Fund	.392
F. R. Lillie Memorial Fund	6.824
Lucretia Crocker Fund	7.391
E. G. Conklin Fund	1.252
M. H. Jacobs Scholarship Fund	.889

During the year we received a gift entitled "Frank R. Lillie Fellowship Fund" consisting of 800 shares of Crane Company common stock. While we can change the investments in this account, it must be kept intact. It was therefore not eligible to be included in the Pooled Fund account. Accordingly, a special custodian account was established for these securities. We are also holding in this account the securities of the General Biological Supply House.

The pledge of $8,000 par value government bonds still remains to secure the loan of the MBL Club which has been reduced over the years from $7,000 to $2,330 at the year end.

Donations from MBL Associates for 1956 were $5,255.00 as compared with $2,940.00 for 1955. Gifts from individuals for unrestricted use were $679.00. Foundations, societies and companies donated $25,500.00. The $6,677.83 balance of the $50,000 from the National Science Foundation for hurricane rehabilitation has been used to cover delayed repairs to the plant, attributed to the hurricane.

The apartment house rentals during the winter months amounted to $2,902.30. The cost of heating was $922.19 leaving a balance of $1,980.11 to be applied against the general expenses of lighting, water and insurance.

We were able to purchase short term treasury bills to activate some of the cash in the Falmouth account arising from the donations from foundations for anticipated construction, the payment for which was scheduled at a later date. The purchase of $50,000 U. S. Treasury bills was made in November, 1956 to run for 90 days. The interest earned on this purchase was $330.50.

Lybrand, Ross Bros. and Montgomery have examined our books and submitted financial statements for examination.

Following is a statement of the auditors.

To the Trustees of the Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheets of the Marine Biological Laboratory as at December 31, 1956, and the related statements of operating expenditures and income and of current fund for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such test of the accounting records and such other auditing procedures as we considered necessary in the circumstances.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of the Marine Biological Laboratory at December 31, 1956, and the expenditures and income for the year then ended.

LYBRAND, ROSS BROS. & MONTGOMERY

Boston, Massachusetts

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1956

Investments

Investments held by Trustee:	
Securities, at cost (approximate market quotation $1,472,265)	$ 995,086
Cash	1,552
	996,638
Investments of other endowment and unrestricted funds:	
Pooled Investments, at cost (approximate market quotation $242,759)	223,341
Other investments (Note A)	75,085
Cash	6,690
Accounts receivable	6,838
	311,954

Plant Assets

Land, buildings, library and equipment (Note B)	2,424,492
Less allowance for depreciation (Note B)	991,930
	1,432,562

Current Assets

Cash	100,045
U. S. Treasury bills, face value $50,000, due 2/15/57 temporarily invested pending expenditures for rehabilitation of Supply Department building and certain laboratories (approximate market quotation $49,800)	50,000
Common stocks, at market value at date of gift	5,728
Accounts receivable ($12,880 from U. S. Government)	25,535
Inventories of specimens and Bulletins	58,269
Prepaid insurance and other	15,980
	$2,996,711

Notes:

A—The Laboratory has guaranteed a note of approximately $2,400 of the M. B. L. Club and has pledged as security therefor bonds with an original cost of $7,900 included in other investments.

B—The Laboratory has since January 1, 1916 provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1956

Endowment Funds

Endowment funds given in trust for the benefit of the Marine Biological Laboratory		$ 996,638
Endowment funds for awards and scholarships:		
Principal	$ 62,861	
Unexpended income	1,420	64,281
Unrestricted funds functioning as endowment		206,378
Retirement fund		41,824
Pooled investments—accumulated gain or (loss)		(529)
		311,954

Plant Liability and Funds

Mortgage payable on demand, 5%		5,000
Funds expended for plant, less retirements	2,419,492	
Less allowance for depreciation charged thereto	991,930	1,427,562
		1,432,562

Current Liabilities and Funds

Accounts payable	13,653
Unexpended balances of gifts for designated purposes	11,540
Advance payments on research contracts	83,189
Current fund	147,175
	$2,996,711

MARINE BIOLOGICAL LABORATORY

Statement of Operating Expenditures and Income

Year Ended December 31, 1956

Operating Expenditures

Direct expenditures of departments:	
Research and accessory services	$161,259
Instruction	33,186
Library, including book purchases	28,631
Biological Bulletin	15,902
	238,978
Direct costs on research contracts	55,343
Administration and general	48,744
Plant operation and maintenance	76,926
Hurricane emergency repairs	6,678
Dormitories and dining services	131,464
Equipment purchased from current funds	2,850
	560,983
Less depreciation included in plant operation and dormitories and dining services above but charged to plant funds	36,424
	524,559

Income

Direct income of departments:	
Research fees	44,507
Accessory services (including sales of biological specimens $66,571)	94,978
Instruction fees	17,174
Library fees and income	6,625
Biological Bulletin, subscriptions and sales	18,148
	181,432
Reimbursement and allowance for direct and indirect costs on research contracts	56,429
Dormitories and dining services income	108,375
	346,236
Investment income	81,501
Gifts for current use	124,062
Sundry income	376
Total current income	552,175
Excess of income	$ 27,616

MARINE BIOLOGICAL LABORATORY

STATEMENT OF CURRENT FUND

Year Ended December 31, 1956

Balance January 1, 1956	$119,559
Excess of income over operating expenditures, 1956	27,616
Balance December 31, 1956	$147,175

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1956

	Cost	% Total	Approximate Market Quotations	% of Total	Investment Income 1956
Securities held by Trustee:					
General endowment fund:					
U. S. Government bonds	$ 97,052	11.7	$ 93,462	7.7	$ 4,299
Other bonds	406,226	48.9	409,437	33.6	8,990
	503,278	60.6	502,899	41.3	13,289
Preferred stocks	85,788	10.3	71,500	5.8	3,370
Common stocks	241,652	29.1	644,556	52.9	29,350
	830,718	100.0	1,218,955	100.0	46,009
General Educational Board endowment fund:					
U. S. Government bonds	31,037	18.9	30,164	11.9	1,096
Other bonds	65,925	40.1	53,200	21.0	1,276
	96,962	59.0	83,364	32.9	2,372
Preferred stocks	27,281	16.6	24,274	9.6	1,130
Common stocks	40,125	24.4	145,672	57.5	5,807
	164,368	100.0	253,310	100.0	9,309
Total securities held by Trustee	$995,086		$1,472,265		$55,318

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS—*Continued*

December 31, 1956

	Cost	% Total	Approximate Market Quotations	% of Total	Investment Income 1956
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government bonds	$ 37,800	16.9	$ 34,119	14.1	$ 905
Other bonds	93,936	42.1	90,628	37.3	2,527
	131,736	59.0	124,747	51.4	3,432
Common stocks	91,605	41.0	118,012	48.6	5,439
	223,341	100.0	$ 242,759	100.0	8,871
Other investments:					
U. S. Government bonds	7,920				220
Common stocks	43,600				20,613
Real estate and mortgage	23,565				
	75,085				20,833
Total investments of other endowment and unrestricted funds	$298,426				$29,704
Total investment income received					85,353
Custodian's fees charged thereto					(412)
					$84,941

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THE BREEDING OF POLYCHAETOUS ANNELIDS NEAR PARGUERA, PUERTO RICO

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The writer set out to accomplish an embryological problem involving some of the polychaetous annelids of Puerto Rico. No information on the breeding habits of this group was available. To obtain suitable embryological material, therefore, it was necessary to determine the breeding periods of some of the species which were readily accessible. The information obtained is thus incidental to the main problem. Several people, however, have expressed an interest in its publication, since apparently little is known concerning the breeding habits of tropical marine forms. The information may be of some value to others interested in studying tropical or subtropical marine forms.

Observations and collections were made throughout the year. Night collections were made at the laboratory dock on the island of Magüeyes, Parguera, Puerto Rico, with a reflector bulb attached to an extension cord, and a dip net, or from a boat over nearby coral reefs with the aid of a reflector light attached to a battery. Some of the former collections were made throughout a full year; collections over the reefs were made from March to December, 1955. Collections made during the day were largely from the shallow seas over coral reefs, and along the edge of mangrove islands. The waters of this area of the Caribbean are relatively warm throughout the year, being somewhat cooler during the "winter" months. The temperature of the surface water goes as high as 87° F. at the laboratory dock (September 1, 1955). The tides in this area show little variation in height, the difference between the lowest low tide and the highest high tide rarely exceeding 41 cm. in any month.

Dr. Olga Hartman and Dr. Marian H. Pettibone were kind enough to identify the species here described. I am most grateful for their generous help, and also wish to express my appreciation to Mr. Donald Erdman who kindly assisted with many of the night collections, and to those individuals of the College of Agriculture who were so cooperative in putting the facilities of the Marine Institute and of the Department of Biology at my disposal.

The polychaetes on which observations have been made are arranged by families for convenient reference. Throughout the paper, one asterisk (*) refers to those species identified by Dr. Hartman, and two asterisks (**) to those identified by Dr. Pettibone.

FAMILY SYLLIDAE. *Autolytus ornatus* (Verrill) *. The female or sacconereis stage of the red-banded *Autolytus* may be collected at the dock at night the year round with the possible exception of the end of January and the beginning of February. It was found to be most abundant during the last half of May and June, during July (particularly during the middle of the month), and the first part of September. Individuals rise from a depth of several feet to the surface within the circle of light. When abundant, as many as 50 have been collected with one dip of the net, and as many as 165 have been collected within an hour. The female stage of this polychaete measures approximately 13 mm. However, individuals appear about half this size as they tend to curl ventrally around an egg sac in which developmental stages are borne. Before being released, white spherical eggs, approximately 85 micra in diameter, are packed within the coelom for the entire length of the body. Their development will be considered in a subsequent paper. The male (polybostrichus) stage of this syllid has been observed only twice. This was not at the dock where the sacconereis stage collects but over a reef on the nights of July 26, when 8 specimens were obtained, and September 21, 1955, when 2 specimens were collected.

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In a series of papers written in 1951, Fauvel describes some polychaetous annelids from the Gulf of Tadjoura (French Somaliland) caught by M. J.–L. Dantan during night fishing with a light in the months of January, February, and March, 1933 and 1934. The Gulf of Tadjoura is closer to the equator than is Puerto Rico so presumably the water would be very warm the year round. Fauvel (1951a) notes that during night fishing in the gulf the syllids and nereids were the most abundant and most interesting of the species collected although other epitokal species were obtained too. The collecting in Puerto Rico also yielded primarily syllids and nereids. Fauvel (1951b) describes a number of species of syllids including *Autolytus.* It is interesting to note, however, that the polybostrichus stage of *Autolytus* was caught in enormous quantities (many hundreds) while the sacconereis stage was relatively rare (Fauvel, 1951a). As described above, the reverse was true in the Puerto Rican waters (although "enormous quantities" were not obtained), the male stage rarely being observed. From a plankton collection Thorson (1946) reported three sacconereis specimens of *A. prolifer* with larvae close to hatching stage in the ventral egg sac and one polybostrichus stage of the same species.

The temperature of the surface water from which *A. ornatus* was collected at Parguera, Puerto Rico, probably never goes below 75° F., even in January. It is interesting to note Bumpus (1898a) has reported that at Woods Hole, Massachusetts, during the month of March, 1898, *Autolytus cornutus* was frequently taken with eggs, the temperature ranging from 38° F. at the beginning of March to 43° F. at the end. Mead, in 1898, reported that *Autolytus* (species unspecified) with egg clusters attached was regularly taken in his tow at Woods Hole, usually 3 or 4 at a time, during the early part of April (water temperature approximately 41 to 42° F.). Bumpus (1898b) noted that the tow-net on May 7 brought in *Autolytus* but without eggs; on May 10th he noted several egg-bearing individuals, and on the 11th a male swimming about and finally fastening to a female with his jaw. Throughout the latter portion of May, *Autolytus* was abundant. In his report for June, July, and August, Bumpus (1898c) notes that *Autolytus* and other syllids which were frequently taken at Woods Hole had ventral egg sacs.

In the cold waters of Point Barrow, Alaska, both female stolons (with egg sacs) and male stolons have been reported for several species of *Autolytus* (Pettibone, 1954). This material was collected by Dr. G. E. MacGinitie of the Arctic

Research Laboratory. While the writer was still collecting *A. ornatus* in Puerto Rico, the following very interesting description of *Autolytus fallax* from the Point Barrow collection was published (MacGinitie, 1955, p. 138): "The female sacconereis stages bearing egg sacs were taken through the ice from January 25 to May 17, 1950. On March 29, 1950, there were hundreds of these worms, with egg sacs, swimming in the water at the 80-foot plankton hole and on April 7, 1950 (1.9 miles from shore) when the slush ice was removed from a 4-foot-wide lead, hundreds were welling up and swimming around. The worms were about 10 mm. long and the egg sacs 2.5 mm. long." It thus appears that *Autolytus* is able to tolerate a very wide temperature range for breeding, and that in the warm waters of Puerto Rico *A. ornatus* breeds all the year round with the possible exception of late January and early February. Hartman (1951) remarks that both sacconereis and polybostrichus stages of *Autolytus brevicirrata* Winternitz may be taken in plankton during the summer. Dales' comments (1951) on the breeding season of *A. prolifer* (*A. cornutus?*—see Pettibone, 1954) indicate that the breeding habits of this species may be similar to those of *A. ornatus*.

The sacconereis stage of another *syllid*, unidentified as yet, was collected occasionally along with *A. ornatus*. This species is yellow in color, is not banded, and has smaller eggs and larvae (various stages were collected) than those of *A. ornatus*. Thus far it would appear that this species cannot be collected in sufficient numbers for embryological purposes.

FAMILY NEREIDAE. *Nereis riisei* Grube*. Both male and female heteronereids of this species have been obtained in the evenings, both at the dock and over reefs, during most of the year. No real swarming, such as has been described for some species of *Nereis*, has been noted. Usually only one to a few individuals have been observed in an evening. The largest number observed was within an hour's time (between 8:00 and 9:00 P.M.) on March 23. Shedding does not ordinarily occur when the worms are isolated but only after a male and female are put together. The spherical eggs, approximately 190 micra in diameter, have numerous oil droplets as is characteristic of other nereid eggs. Results following attempts at artificial fertilization varied. In some cases no shedding occurred; in others, shedding of gametes occurred without development; in others, development ceased during cleavage; and in successful cases the eggs developed into larvae with three external sets of setae within three days. Results suggest that the height of the breeding period of this form extends from the last week in July to the beginning of September. None were obtained during four observations throughout June, with the exception of June 27 when one individual was observed. None were observed during the last part of September and October, 1955 (a few were collected on October 7, 1954). One was observed on November 7, none on November 14, and two (one with posterior end missing) on November 22, 1955. Occasional individuals were collected throughout the other months of the year.

A number of nereids have been described by Fauval (1951a-d) from the collection from the Gulf of Tadjoura but this group did not include *Nereis riisei* Grube. The heteronereid stages of *N. riisei*, collected at Parguera, Puerto Rico, are in size, color, and behavior under the light, reminiscent of *Nereis succinea* (Leuckart), commonly called *Nereis limbata* (Ehlers), found at Woods Hole, Massachusetts. *N. riisei* differs, however, in that the males observed were larger than the females and no real swarming occurs. This is in even greater contrast to the spectacular behavior of the *Nereis succinea* occurring in eastern Canadian waters (Berkeley and Berkeley, 1953). Swarming in the case of *N. succinea* (at least, in the Woods Hole area) seems to be more closely associated with the lunar cycle (Lillie and Just, 1913) than is the appearance of the heteronereid stage of *N. riisei* in Puerto Rican waters.

Ceratonereis mirabilis Kinberg **. Small heteronereids of this species (approximately 7 mm. in length) were collected at night over coral reefs. They were never observed at the dock. Night collections over reefs were begun on March 11, 1955, when the only suggestion of real swarming of this species was observed. The heteronereids shed their gametes when isolated. Males appear yellow in color prior to shedding and pinkish (posterior portion) after shedding. Females appear whitish before and pinkish after shedding. The eggs, approximately 120–125 micra in the long diameter, are flattened spheres suggesting biconvex discs. Artificial fertilization was attempted whenever both males and females were collected. It was always successful. Embryos swim within 8 hours after fertilization, trochophores develop within 12 hours after fertilization, and beautiful larvae with three bushy sets of setae develop within 2½ days. A few have been kept alive for 22 days but developed no additional setae. Collections were made from March 11 to December 14, 1955, but both sexes were observed only during the new moon each month with the exception of March 11, three days after full moon, when the best batch of eggs was obtained (to date this exception has not been explained), and three days before the first quarter in September when a few were obtained. It should be noted that only one sex in the heteronereid form was collected during the new moons of June, September, and December.

Fauvel (1951c) describes several species of *Ceratonereis,* including *C. mirabilis,* from the night collections in the Gulf of Tadjoura. In several species, only heteronereid males were caught. In the case of *C. mirabilis,* the male epitoke was caught in numbers with a few females (mostly in fragments). Fauvel points out that while this species is very widely distributed in all warm regions of the Atlantic, of the Pacific and particularly of the Indian Ocean (with which the Gulf of Tadjoura is indirectly connected), the epitokal form has been only rarely encountered, the few females being mainly fragments. In the Puerto Rican waters the writer was apparently fortunate in obtaining both sexes in the heteronereid form for embryological purposes.

*Nereis sp.***. This is a small pink species, measuring approximately 2 cm. in length, with a rather thick body. This worm was frequently collected along with *Ceratonereis* and other small nereids, but insofar as can be determined only the male heteronereids were observed. This has been the experience of other investigators (see Fauvel, 1951a-d).

Platynereis dumerilii (Audouin and M. Edwards) **. This small pink heteronereid, flattened dorso-ventrally, is usually somewhat smaller than the preceding. The heteronereid stage was collected frequently along with *Ceratonereis* and *Nereis sp.* Again, only male heteronereids were detected. Fauvel (1951d) describes several species of *Platynereis* but not *P. dumerilii.* It is interesting to note that in the *Platynereis dumerilii* at Naples which breeds from October through May spectacular swarming has been observed after the full moon in May (Just, 1929), while only a few heteronereids were observed in Puerto Rico and these during the last quarter of the moon and the new moon (several observations). The heteronereid stages of *P. dumerilii* were also observed between 8:00 and 11:00 P.M. in March, 1955, during the dark of the moon (no moon) on Loch Hourn, Scotland (Dr. L. R. Fisher, personal communication, 1957). These observations suggest that *P. dumerilii* is able to tolerate a wide temperature range for breeding.

Nereis allenae (Pettibone). Only two heteronereids of this small species (approximately 1 cm. long) have been collected, one on August 25 (first quarter of the moon) and one on September 15 (new moon), 1955. Both were collected during the evening from the shallow water over the reef between the Marine Laboratory and Caballo Blanco Island, Parguera, Puerto Rico. This was the first time that the writer had observed polychaete eggs laid in short strings resembling blue-green algae. The eggs made up a single row of cells (approximately 30 eggs in some strings). Within about an hour after laying, the eggs became isolated due to the dissolution of the substance (jelly?) holding the eggs together. The eggs after fixation measure approximately 160 micra in diameter. This polychaete is a new species (Pettibone, 1956).

Several other species of nereids are represented by heteronereids (including egg-laying females) in night collections but they have not, as yet, been identified.

FAMILY GLYCERIDAE. *Glycera ?sphyrabrancha* Schmarda **. On the evening of October 31, 1955, between 8 and 8:40 P.M., two females, 28 cm. and 23 cm. long, respectively (when fixed), and a long male segment of this species were observed swimming in surface water at the dock light. They were caught in a dip net and isolated in finger bowls, whereupon the shorter female and the male segment shed their gametes. The eggs (resembling flattened tops and approximately 125 micra in diameter) are whitish, with a large central germinal vesicle (50 micra in diameter) and finely granular cytoplasm. They are liberated in two streams, apparently from two pores about midway down the body. Two drops of diluted very active spermatozoa were used to inseminate the eggs. At approximately 9:30 P.M. another female was observed and caught with the dip net. The relatively few eggs suggested she may have shed already. Within 1 hour and 40 minutes after insemination a number of the eggs of the first female were at the 2- to 4-cell stage (mostly the latter) but a number had not cleaved. Within 11 hours many ciliated swimmers were observed in the dish. Within the next 24 hours larvae with a prominent prototroch had formed and within the next 24 hours (age, 2½ days) elongating larvae with a differentiating gut had developed.

EUNICIDAE. *Eunice ?pennata* (Müller) **. These slender worms, approximately 7½ cm. long, build small sand tubes, externally covered with shell. They are found on the under surface of coral in shallow water. When the coelom of a female is packed with eggs, the posterior half of the body appears green due to the pigment within the eggs. The eggs, approximately 175 × 160 micra in diameter, are oval with a prominent germinal vesicle. The posterior half of males with spermatozoa appears cream in color. Not many specimens have been collected, but females with eggs have been observed in February, March, April, May (one female was observed over a reef on the evening of May 6), September, and November, 1955. The few attempts at artificial fertilization were unsuccessful.

*Eunice (Nicidion) sp.***. This is a small species about the size of *E. ?pennata*. The worms are found on the under surface of coral in association with *E. ?pennata* tubes, or close to them, and may occur in clumps of several individuals twined about one another. The eggs (observed only in September) are bright pink and may be observed readily through the body wall. This species has been observed only in September, October, and November, but no definite attempts were made to look for it. Fauvel (1951d) identified several species from the Gulf of Tadjoura collection, including *Eunice* (*Nicidion*) *edentulum* which he describes as a cosmopolitan and widely distributed species living in the Indian Ocean, Pacific, and Atlantic.

*Lysidice sp.***. Between 7:30 and 9:00 P.M. on the evening of December 14, 1955, a number of worms were observed in the circle of light swimming in the shallow water over a reef near the laboratory and several were shedding gametes. The adults were isolated and the eggs were inseminated artificially. In about three hours spherical surface swimmers were observed and 16 hours after insemination orange-red eyespots were visible. This species also was collected in November but shedding was not observed. Fauvel (1951d) identified *Lysidice collaris* from the Gulf of Tadjoura and described it as being widely distributed in warm seas.

AMPHINOMIDAE. *Hermodice carunculata* (Pallas) *. This large fireworm is rather plentiful in the Puerto Rican area and is locally referred to as a "marine centipede." It has been observed during the daytime lying about on reefs in shallow water or on the sandy to muddy bottoms surrounding mangrove islands. An occasional individual was checked for gametes at least once a month from January through June, 1955, by making a small slit in the body wall. No gametes were observed in this period except on January 13 when an injured female shed many orange-colored eggs, approximately 100 micra in diameter. In November, 1955, a few specimens were again checked for gametes. Eggs were observed (though not very abundant) in one female on November 8, and in another on November 29.

Eurythoë complanata (Pallas) *. This is another common "marine centipede" often collected along with *H. carunculata* on shallow reefs. Eggs have not been observed but very small worms have been collected early in November (one measured 19 mm. and a second 13 mm. when fixed). Fauvel (1951a) identified one specimen of this species taken from the Reef of Ambouli, noting that it is found in all tropical seas. Both this species and the preceding are found in the Gulf of Mexico along the coast of Florida (Hartman, 1951).

Notopygos crinita Grube **. This species of fireworm has been observed only in May. On the evening of May 13, 1955, two males and one female were observed swimming over a reef. When isolated in finger bowls, they shed immediately. The eggs fertilized readily, developing into much flattened, top-like trochophores within 14 hours. Four days after insemination, they had developed into weird-looking larvae with two sets of much elongated glass-like setae. The larvae died within the next two days. On the evening of May 20 a small male was collected similarly. It did not shed until taken to the laboratory. No other individuals were observed.

FAMILY POLYNOIDAE. *Chaetacanthus magnificus* (Grube) *. Often one to several of these worms were collected incidentally on coral reefs in shallow water from September, 1954, to May, 1955, but no shedding was observed nor were gametes seen when the body wall was pricked. Later (November 14, 1955) a few more were collected. Upon puncturing the body wall one of these shed a few whitish, opaque, almost spherical eggs, approximately 100 × 85 mm. in diameter.

FAMILY TEREBELLIDAE. *Thelepus setosus* (Quatrefages) *. This species is found in tubes, covered by sand and shells, on the under surface of coral in shallow water. Pink, disc-shaped eggs (the larger measuring approximately 185 micra in diameter) are readily observed through the body wall moving about within the coelomic fluid. A number of individuals have been collected each month from November, 1954, to November, 1955, at various phases of the moon (collections were made during the day) but no fertilization has been attained. Individuals when collected were isolated and checked periodically for shedding. Changing the temperature, and also the sea water in which they have been kept, was done consistently but did not prove successful in stimulating shedding. In one instance, on March 31, a male shed active spermatozoa within 30 minutes after isolation; an attempt at artificial fertilization was unsuccessful. On September 10, one individual out of six shed many eggs on the day after it was collected (following two changes of temperature and of sea water). The breeding habits of *Thelepus* may be similar to those of its relative, *Amphitrite ornata* Verrill. Mead (1897) collected approximately 800 specimens of *A. ornata* from June through August and only rarely obtained ripe gametes. He stated (p. 229) that it "is useless to cut the animals open, for, if the sexual products are mature, they will be discharged, usually at about 6 o'clock in the evening, more often on the day of capture, sometimes the next day. The rarity of ripe specimens is partly compensated for by the enormous number of eggs which may be obtained from one female." Scott (1909) concluded that the height of spawning for *A. ornata* is in July and is closely related to the spring tide. As suggested by the above observations, no definite conclusions can be drawn, as yet, regarding the specific breeding habits of *Thelepus setosus*.

Eupolymnia crassicornis (Schmarda) *. This worm lives in a collapsible tube of mucus reinforced by pebbles, sand, shell, calcareous algae, etc. The breeding habits of this species probably are similar to those of *Thelepus*. Eggs, if plentiful enough, appear as lavender areas seen through the body wall. The color is due to the pigment in the ova. The larger of the disc-shaped eggs are approximately 175 micra in diameter. This species likewise has been checked the year round, from January to December, 1955, and treated in the same manner as *Thelepus*. On March 15, at 5:00 P.M., individuals collected that day were isolated, and by 8:00 P.M. two females had shed many eggs, and one a few eggs. By then, the germinal vesicle had broken and one male had shed active spermatozoa. Two batches of eggs were inseminated; one showed no development and the second had an occasional swimmer moving slowly within 3½ hours after insemination. All of these swimmers died within the next day and a half. Possibly the eggs were over-ripe. On June 27, another attempt at artificial insemination was unsuccessful. On September 11, six males shed active spermatozoa. Artificial insemination was tried although the female used had not shed normally. No development occurred. On November 15, one out of the six worms collected that morning had shed active spermatozoa by 6:00 P.M., and on November 29 one of two males shed at approximately 6:00 P.M.

SABELLIDAE. *Sabellastarte magnifica* (Shaw) **. This large feather duster lives in a membranous tube attached to coral in shallow water. Relatively few specimens have been checked the year round for gametes. The rather small eggs (approximately 55 micra in diameter) are whitish discs with a central germinal vesicle. They tend to shrink when freed from the coelom, suggesting that they are not osmotically balanced with the sea water. Eggs have been observed in August, September, and October, and males with active spermatozoa have been collected in September, October, and November (one male, November 23). The few attempts at artificial fertilization were unsuccessful.

Sabella melanostigma Schmarda*. This species of feather duster, smaller and more slender than the preceding, lives in a slender membranous tube (covered with fine silt and mud) found attached to stones or shells or to mangrove roots. Individuals with eggs (most of the eggs shrivel in sea water) and motile spermatozoa were collected from January through May. No gametes were observed in the few specimens checked in June, July, and August. One check was made in September — two females examined had minute eggs; a male individual, considerably smaller than the females, had active spermatozoa. Both eggs and active spermatozoa were noted in October and November. The few attempts at artificial fertilization, scattered throughout the year, proved unsuccessful.

SERPULIDAE. *Spirobranchus tricornis* (Mörch) *. This small feather duster builds a calcareous tube in the common coral, *Porites porites*. The eggs, approximately 80–85 micra in diameter, are flattened spheres. They are relatively clear except for the orange pigment granules in the cytoplasm. The sex of individuals can often be determined with the naked eye if the coelom is packed with gametes. Females have bright orange tips (basal end), as the pigment in the mass of eggs shows through the body wall; males have whitish or creamish tips when packed with spermatozoa. This species has been checked for gametes in every month from September, 1954, to November, 1955. Only a few swimmers developed following attempts at artificial fertilization from November through February. A number cleaved following artificial fertilization from March through October, 1955. These developed into typical trochophores, some batches swarming with the ciliated swimmers. A member of this genus, *Spirobranchus giganteus* (Pallas), has been described by Fauvel (1951d) in the collection from the Gulf of Tadjoura. He notes that this species occurs in all intertropical regions of the Indian Ocean, Pacific, and Atlantic, mainly on coral reefs.

It is hoped that the positive results recorded here will prove useful to other investigators interested in tropical or subtropical marine forms. Negative results should be viewed with caution.

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ENCYSTMENT STAGES OF DICTYOSTELIUM [1]

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The genus *Dictyostelium* Brefeld (1869) is representative of the Acrasieae, a group of simple, cellular slime molds, wherein the life cycle consists of an amoeboid vegetative phase and a plant-like fruiting phase. The vegetative phase of these primitive micro-organisms is characterized by the independent movement and multiplication of free-living myxamoebae which feed by the ingestion and digestion of bacterial cells. The fruiting phase normally begins with the exhaustion of this food supply and is first evidenced by the coordinated inflowing of the myxamoebae to form wheel-shaped aggregates, or *pseudoplasmodia*. It attains its definitive expression as a portion of the myxamoebae thus assembled becomes transformed into vacuolate, parenchyma-like cells to form the upright stalk, or *sorophore,* whilst the remainder differentiate into capsule-shaped reproductive cells, or *spores,* to form the elevated spore mass, or *sorus,* of the slime mold fructification, or *sorocarp*. In most cultures of *Dictyostelium* grown under favorable cultural conditions, virtually all of the former vegetative myxamoebae enter directly into radiate pseudoplasmodia and subsequently differentiate into either stalk cells or spores (Olive, 1902; Raper, 1935, 1940a, 1940b; Bonner, 1944). However, marked exceptions to this behavioral pattern occur in certain species and strains.

Under some conditions, not wholly understood, many of the vegetative myxamoebae never enter the fruiting state (*i.e.*, aggregate to form pseudoplasmodia) but as individual cells enter an encystment stage. Such resting cells are termed *microcysts* (Fig. 1). This designation was first applied by Cienkowski (*fide* Olive, 1902) to describe those myxamoebae of *Guttulina* which under unfavorable conditions tended to form rounded protoplasmic bodies with definite ectoplasmic membranes. Olive widened the application to include other Acrasieae and stated that microcyst formation occurred under unfavorable conditions such as slow drying in hanging-drop preparations. In our investigations such individually encysted cells have been observed from time to time and in large numbers in agar plate cultures of *Dictyostelium mucoroides* Brefeld (1869), and with even greater frequency in *D. minutum* Raper (1941), *D. polycephalum* Raper (1956a, 1956b), *Polysphondylium pallidum* Olive (1901, 1902), and *Acytostelium leptosomum* Raper (1956b). They are believed to occur in greater or lesser numbers in all aging cultures.

An additional, multicellular encystment stage occurs in occasional strains of *Dictyostelium mucoroides* and in many isolates of *D. minutum*. These relatively complex structures arise by a morphogenetic process possibly alternative to normal

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sorocarp formation. Because of their larger dimensions and their multicellular origin and constitution, these bodies are termed *macrocysts* (Fig. 2). This designation was first applied by Raper in 1951 with reference to structures seen in certain cultures of *D. minutum* isolated from soil, but no description of the macrocysts was given. More recently, Cormier and Raper (1955) and Raper (1956b) have reported briefly concerning their formation and structure. It is of special interest that Brefeld, in his original paper on *D. mucoroides* (1869), described and illustrated as "dwarfed sporangia" structures which are believed to have represented macrocysts (Fig. 3).

The primary purpose of this investigation has been to extend our observations on these long neglected structures and to seek answers to three basic questions pertaining to them, namely: How are the macrocysts and microcysts formed? What conditions favor their development? What are the roles of these encystment stages in the life cycle of the Acrasieae? A resumé of our present knowledge of these matters is presented herewith.

MATERIALS AND METHODS

Micro-organisms investigated

Several cultures of *Dictyostelium* were examined relative to the production and possible function of the macrocysts, including *Dictyostelium mucoroides* (Strains S–28b, NC–12, and WS–47) and *D. minutum* (Pur–8a and WS–56–2). *Dictyostelium mucoroides* was the slime mold studied most intensively, particularly strain S–28b which produces abundant macrocysts. Macrocysts were first observed and photographed in strain NC–12 when this slime mold was isolated in 1937 (Fig. 2).

Microcysts of several species of the Acrasieae were studied, including those of *Dictyostelium minutum* (WS–116b), *D. polycephalum* (S–4), *Polysphondylium pallidum* (WS–116c), and *Acytostelium leptosomum* (FG–12a). Those of *D. polycephalum* are illustrated in Figure 1.

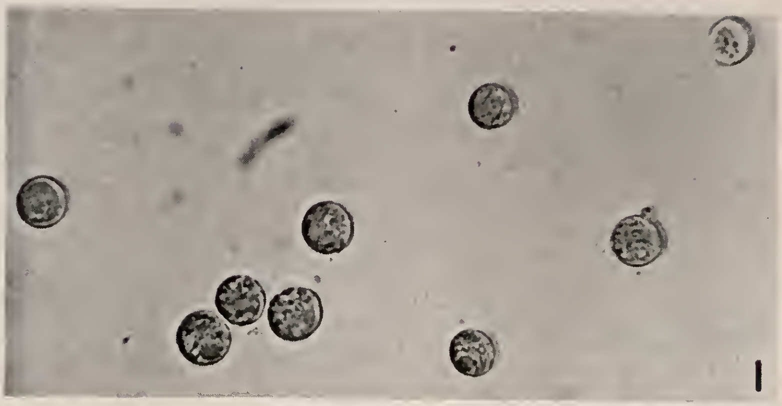
Different bacterial associates were investigated as food sources for the slime molds and for their possible influence upon macrocyst formation. Included among gram-negative species were *Escherichia coli* (No. B–281), *Aerobacter* sp. (Singh's strain), *Aerobacter aerogenes* (Sussman's strain), *Flavobacterium* sp. (DIF), *Serratia marcescens* (No. B–175), and *Pseudomonas fluorescens* (No. B–112); included among gram-positive species were *Bacillus megaterium* (No. B–160), *B. subtilis* (Sarles' strain), and *Sarcina lutea* (No. B–1018). *Escherichia coli,* the organism used most commonly, is a short rod which is mildly proteolytic and ferments both dextrose and lactose; the myxamoebae appeared to grow best when feeding upon this bacterium.

Cultivation of the slime molds

Environmental and cultural conditions, including (1) temperature, (2) culture media, (3) pH of substrate, (4) ammonium ion concentration, and (5) per cent relative humidity, were varied to determine their effect upon macrocyst formation in *Dictyostelium.*

Incubation temperatures used in most of the experiments were 10, 15, 20, 25,

Plate I



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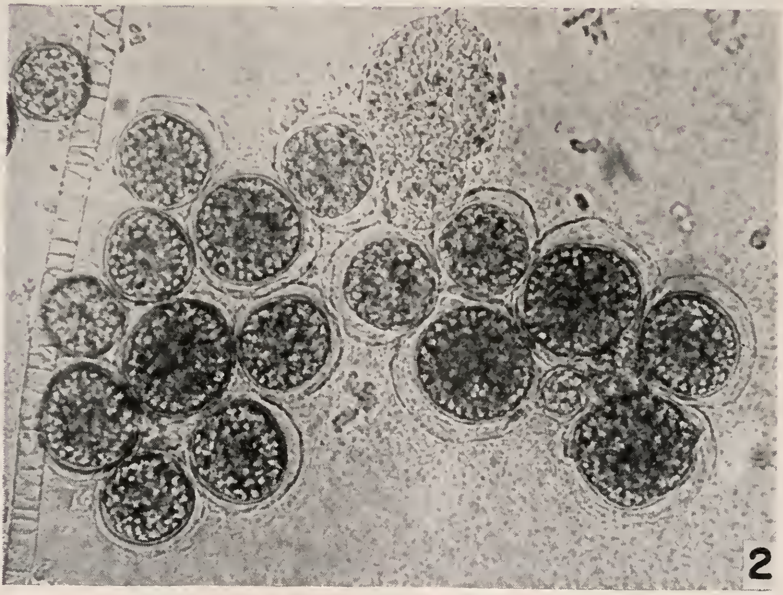

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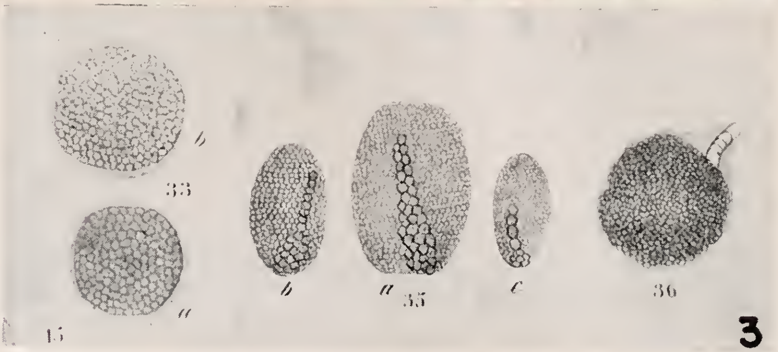

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and 30° C. The optimum growth temperature for the macrocyst-forming strains of *Dictyostelium mucoroides* was about 20° C., whereas that of the *D. minutum* isolates more nearly approximated 25° C.

Many different media were employed for the cultivation of the slime molds and the associated nutritive bacteria, which, for the most part, were patterned after substrates previously reported by Raper (1951). An agar medium containing 0.1% lactose and 0.1% peptone (0.1 L–P agar) was used most extensively since it provided the most reproducible growth and the most consistent macrocyst formation in *D. mucoroides* and in *D. minutum*. Horse dung-infusion and 0.05% uric acid agars[3] were also employed because of Brefeld's report (1869) that dung extract and uric acid induced spore germination.

The pH of the substrates employed for the conjoint growth of the slime molds and associated bacteria was varied by buffering with KCl, potassium acid phthalate, KH_2PO_4, and K_3BO_3 as recommended by Clark and Lubs (Clark, 1928). Bacteria were also pre-grown on buffered 0.1 L–P medium of varied pH and transferred to unbuffered medium prior to inoculation with the slime mold (Raper, 1951). Experiments to determine the possible effect of ammonium ions on growth and macrocyst formation in *Dictyostelium* were patterned after those reported by Cohen (1953).

Different relative humidities were obtained by placing specified concentrations of H_2SO_4 in water in desiccators, as reported by Wilson (1921). Two types of slide cultures were employed: (1) Maximov tissue culture slides containing one ml. 0.1 L–P agar inoculated with a mixed suspension of spores, or myxamoebae and macrocysts, of *D. mucoroides* and *E. coli* cells; (2) plain flat slides spread with 1.0 ml. molten 0.1 L–P agar and inoculated by on-flowing a mixed suspension of slime mold and bacteria. The latter slides were supported in an upright position by small wooden blocks during incubation.

Agar plate cultures were grown routinely in glass Petri dishes, to maintain a high per cent relative humidity, and incubated in the dark at varying temperatures. The plates were cross-streaked or completely smeared with the bacterial associate and then inoculated at the center with spores, macrocysts, or myxamoebae of the selected slime mold, the type of inoculum and bacterial associate being varied with the experiment.

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[3] Dung decoction was made by autoclaving 100 grams of fresh horse dung/liter of water for twenty minutes at 15 pounds' pressure. The resulting decoction was filtered, solidified with 1.5% agar, and re-sterilized (final pH 6.1–6.35). Uric acid agar consisted of a 0.05% aqueous solution of uric acid to which 2.0% agar was added.

PLATE I

FIGURE 1. Microcysts of *Dictyostelium polycephalum*, representing individually encysted vegetative myxamoebae. × 530.

FIGURE 2. Macrocysts of *Dictyostelium mucoroides*, Strain MC–12, showing the characteristic habit and structure of these bodies; the cellular stalk of a normal sorocarp appears at the far left. × 250.

FIGURE 3. Illustrations from Brefeld's description of *Dictyostelium mucoroides* (1869), from left to right: "Dwarfed sporangia," which are believed to be identical with the macrocysts reported in this paper; "small sporangia" with "rudimentary stalks" in the spore-forming plasm; "sporangium" with a small stalk surrounded by an enclosing membrane. × 300.

Reagents

Several reagents were employed to establish the cellulosic nature of the walls of the macrocysts, namely:

(1) Chloroiodide of zinc solution was prepared by dissolving 30 grams of ZnCl, 5 grams of KI, and 0.89 gram of iodine in 14 ml. of distilled water (Stevens, 1916). The material to be tested was mounted in water, after which the reagent was applied to one edge of the cover glass and drawn under it by placing a piece of filter paper against the opposite edge. Cellulosic material stains violet-blue.

(2) Congo red was prepared as a 0.5% aqueous solution of the dye made alkaline by adding two or three drops of concentrated NaOH. In an alkaline solution this reagent stains cellulose red, which when put in HCl turns blue (Raper and Fennell, 1952). This is a presumptive but non-specific test for cellulose.

(3) Schweitzer's reagent was prepared by bubbling air through 60 ml. of NH_4OH containing 10 grams of fine copper turnings for one hour (Hodgman, 1951). Cellulose is quickly dissolved by this strong cuprammonium solution. In practice, the macrocysts were generally mounted in water and the reagent was applied by drawing it under the cover glass, hence diluting and advantageously slowing its action. A 72% aqueous solution of H_2SO_4 was also used as a cellulose solvent.

(4) A strong birefringence in polarized light was likewise interpreted as confirming the predominantly cellulosic composition of the macrocyst wall.

Nile blue sulfate was employed as a vital dye for staining myxamoebae and progressive developmental stages as reported by Bonner (1952). It was also incorporated into 0.1 L–P agar prior to inoculation with *E. coli* and the slime mold, *ca.* 3 ml./liter of a 0.5% aqueous solution being employed for this purpose. Used directly, or when added to the growth medium, it stained vegetative myxamoebae a very light blue, whereas aggregating myxamoebae and the cells of developing macrocysts assumed somewhat darker shades and appeared less granular.

Experimental Results

Origin and morphology of macrocysts

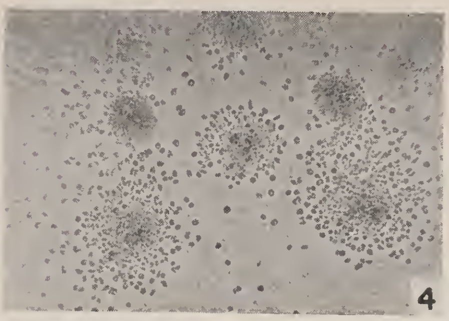
Macrocysts are flattened, irregularly circular to ellipsoidal multicellular structures, ranging from 25 to 50 μ in diameter. The myxamoebae which contribute to their formation appear normal for *Dictyostelium mucoroides* and *D. minutum* in every respect as they move and feed upon bacterial cells, re-dividing until the available food supply is exhausted. As this occurs, the myxamoebae begin to aggregate into pseudoplasmodia which, except for their generally limited dimensions, appear basically similar to other pseudoplasmodia that proceed to sorocarp formation (Fig. 4). However, instead of producing upright sorocarps, the myxamoebae comprising these aggregates remain in compact heaps and subsequently become surrounded by comparatively thick, cellulose walls. When only a few myxamoebae aggregate to form pseudoplasmodia, small macrocysts develop singly; when larger numbers of cells are involved, the macrocysts are somewhat larger and may occur in groups of varying size, ranging from small packets to sheet-like ribbons, depending upon the number of myxamoebae massed together. In these larger aggregations, incipient macrocysts are delimited by the secretion of delicate cellulosic membranes around limited groups of cells more or less regularly spaced throughout the primary aggregate. This is followed by the subsequent deposition of thick, predominantly cellulosic walls, mostly circular to oval in pattern, that become the relatively rigid boundaries of the individual macrocysts. Commonly the secondary wall is laid down in general conformity with the primary membrane, but not infrequently two, three or even more macrocysts develop within an initial area of demarcation (Figs. 5–7).

Somewhat prior to the first evidence of secondary wall formation around the nascent macrocyst, cells in the center of the previously undifferentiated mass exhibit signs of modification and become surrounded individually by strongly refractive membranes. This process of cellular differentiation advances outward until all of the cells comprising the macrocyst are transformed into closely packed, seemingly firm-walled cells, termed *endocytes* (Figs. 8–9). The constitutive myxamoebae show no obvious orientation during the early stages of this process; but as differentiation proceeds, the peripheral and still amoeboid cells become conspicuously elongate as if appressed against the surface of the steadily enlarging body of endocytes (Fig. 6). Parallel with this progressive differentiation of the endocytes, but in a manner not yet understood, the whole body of functionally integrated cells succeeds in building around itself a tough and relatively heavy wall that is rich in cellulose. Significantly, the position of this wall is not determined by that of the thin primary membrane which initially delimited the bloc of myxamoebae that *might* collaborate in macrocyst formation; rather it is determined by the group(s) of cells which first differentiate as endocytes. This is clearly evident from the examination of primary aggregates of different dimensions. If the aggregate is small, the subsequently formed macrocyst wall will conform generally to that of the primary membrane. If the aggregate is relatively large, several centers of endocyte formation will arise simultaneously, and outward from these loci, cells will differentiate progressively to form separate and independent macrocysts, each with its own characteristic heavy wall but all contained within the primary membrane (Figs. 7–9, 25–27). The entire process of macrocyst formation is normally completed within 18 to 24 hours. Successive stages in macrocyst formation are illustrated in Figures 4 through 9.

The endocytes may be isodiametric or slightly elongate, ranging in size from about 3.6 to 4.8 μ in diameter. As observed within the macrocyst, and when first released by breaking the macrocyst wall, these cells normally appear polyhedral in outline, but soon become spheroidal or ellipsoidal when no longer compressed by adjacent endocytes (Fig. 9). For reasons still unknown they are appreciably smaller than the myxamoebae which enter the primary aggregate, the latter usually ranging between 6.0–8.0 μ in the unexpanded state. The smaller dimensions of the endocytes may result from a substantial water loss during their differentiation, or the contributing myxamoebae may possibly undergo division prior to the development of the refractive membranes which so strikingly distinguish them from other cells still amoeboid (Fig. 8).

Endocytes normally remain as distinct cellular entities within the macrocyst for a period of two or three weeks (Fig. 10), after which they commonly lose their identity and the protoplasmic content of the entire structure assumes a homo-

Plate II. Origin and structure of macrocysts in *Dictyostelium mucoroides*, Strain S-28b.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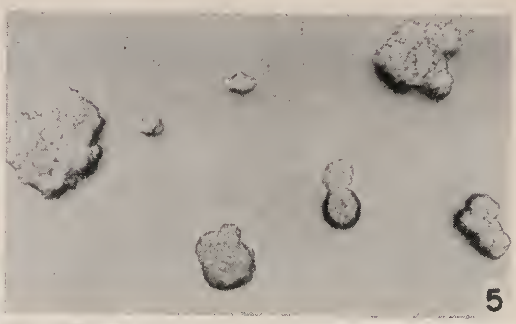
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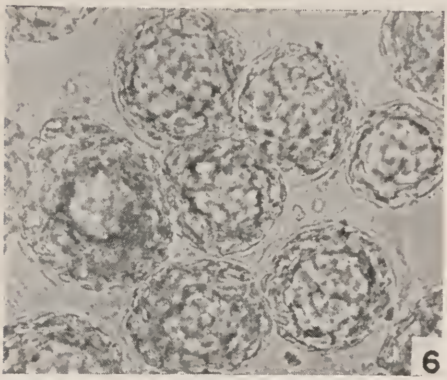
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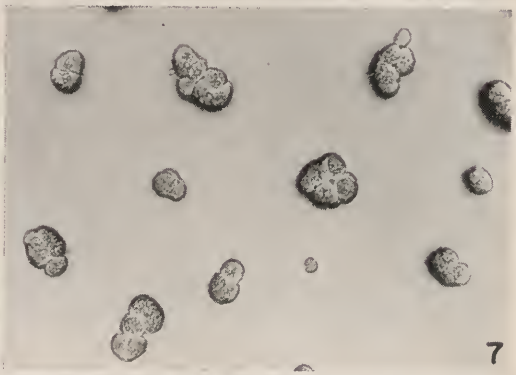
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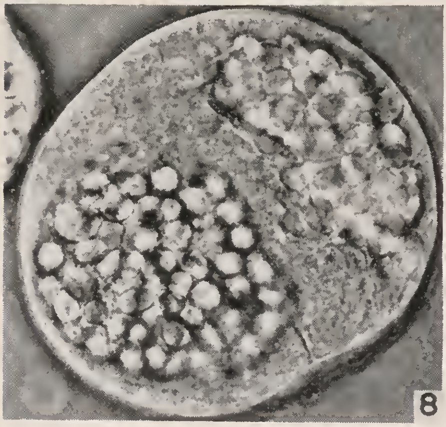
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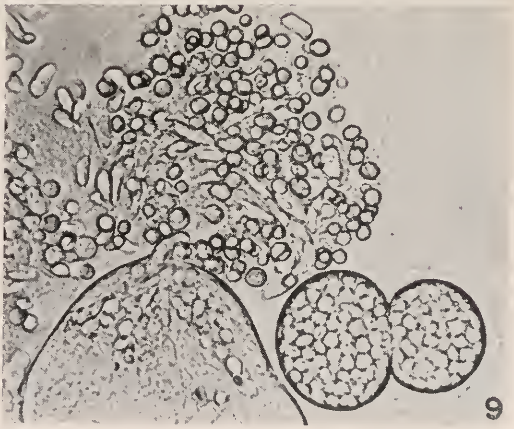
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Figure 4. Small radiate pseudoplasmodia which lead to the formation of macrocysts. × 80.

Figure 5. Completed aggregations, consisting of irregular mounds of myxamoebae, prior to the formation of the primary membranes that delimit incipient macrocysts. × 80.

Figure 6. Enlarged view of a later stage in macrocyst development, showing incipient macrocysts and the orientation of their constituent myxamoebae. × 360.

Figure 7. More advanced stage showing clusters of differentiated endocytes centrally located in developing macrocysts. × 80.

Figure 8. Much enlarged view of two macrocysts in process of formation within a single

geneous appearance. With further aging, from four to six weeks, the apparently acellular content often shrinks to approximately 60–75% of its original volume, and in the form of a compacted, brownish mass occupies a central position within the partially empty macrocyst wall (Fig. 13). The surface of this central body appears slightly irregular and affords no clue to the presence of a continuous bounding membrane; rather it suggests a plasmolyzed and shrunken mass that has been subjected to uneven pressures and tensions during the process of contraction.

Many questions remain unanswered concerning the sequence of events leading to the advanced acellular structures just described. We have inadequate information concerning their true nature, and we have only incomplete knowledge of their significance in the life cycle of those slime molds where they occur. Nevertheless, a detailed study of macrocysts of different ages, and under many conditions, has revealed a considerable body of information concerning their development and behavior. If an endocyte-filled macrocyst is subjected to pressure in an aqueous mount, the heavy cellulose wall breaks, much as a hollow rubber ball, and the endocytes pour out, undergoing the limited changes in shape already noted but retaining their identity as relatively firm-walled cells. In contrast, if an older macrocyst from which the discrete endocytes have disappeared is similarly crushed, the enveloping wall breaks in a comparable manner, but the entire content flows out as a structureless fluid containing innumerable fine particles that immediately exhibit brownian movement as they enter a more aqueous environment.

The explanation for endocyte disappearance in naturally aged macrocysts remains unknown, but a superficially similar state can be produced artificially with alkaline solutions. This was first observed when Schweitzer's reagent was applied to preparations of young macrocysts. Upon contact with the cuprammonium solution, the refractive walls of the endocytes disappeared, the seemingly merged content of the entire macrocyst swelled, and with the partial dissolution of the enveloping cellulose wall, the content emerged as a homogeneous and finely granular mass superficially resembling a large and completely undifferentiated protoplast (Fig. 17). A comparable disappearance of endocytes was subsequently observed in preparations following the application of NaOH (1.0 and 0.1 N), albeit the heavy cyst walls remained intact (Figs. 10–11). The application of HCl (1.0 N) to the same preparations prompted a most dramatic phenomenon, for upon contact with this reagent the endocytes reappeared in their original numbers and positions (Fig. 12). The same phenomenon of endocyte reversal accompanied the application of acid following treatment of young macrocysts with Schweitzer's reagent. If the exposed macrocyst was quite young the endocytes emerged individually as the cellulose wall dissolved, and these collected into a spreading amorphous mass within which cell boundaries remained faintly evident, as seen in Figure 16. If the cyst was older (but still packed with endocytes) the content emerged as an intact, seemingly homogeneous mass following similar treatment (Fig. 17). In each case a discrete cellular structure reappeared with the addition of acid (Fig. 18).

Macrocyst germination has been observed with the emergence of amoeboid cells from the ruptured cyst, and it was first thought that such induced disappearance

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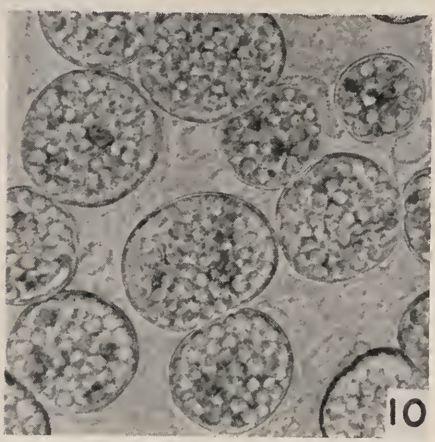
primary envelope, showing clusters of endocytes surrounded by undifferentiated myxamoebae. × 800.

FIGURE 9. A body comparable to that shown in Figure 8, compressed to release endocytes and still undifferentiated cells. × 800.

Plate III. Behavior of young (endocyte-filled) and aged (homogeneous) macrocysts in *D. mucoroides*, Strain S-28b, in the presence of alkali and acid.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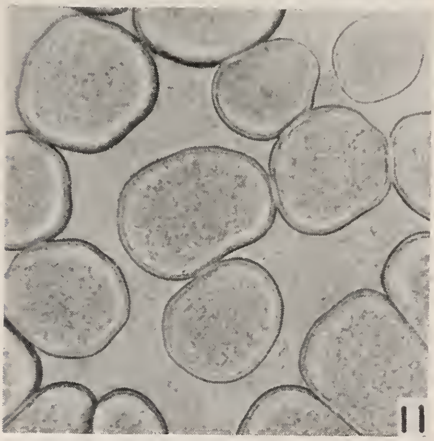
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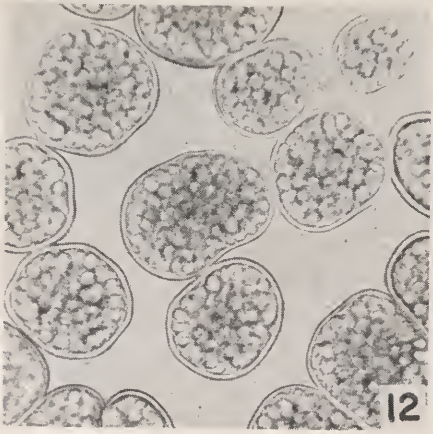
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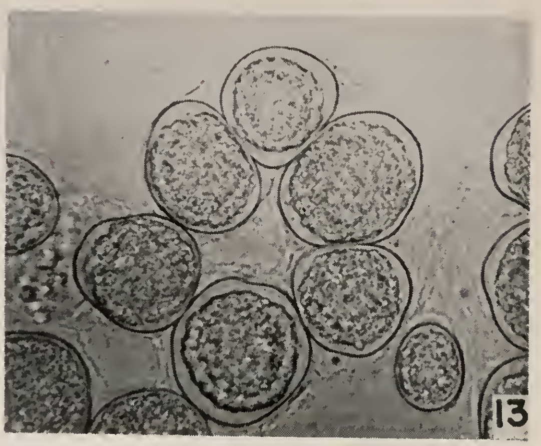
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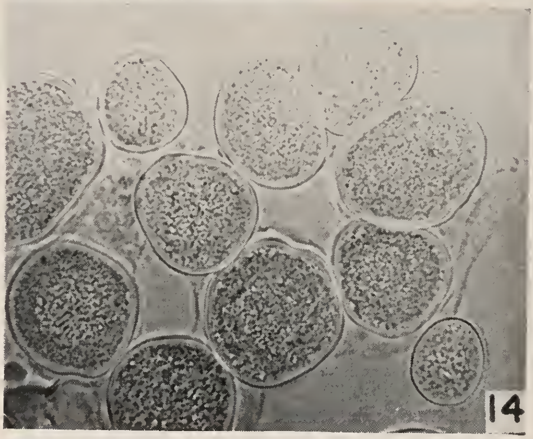
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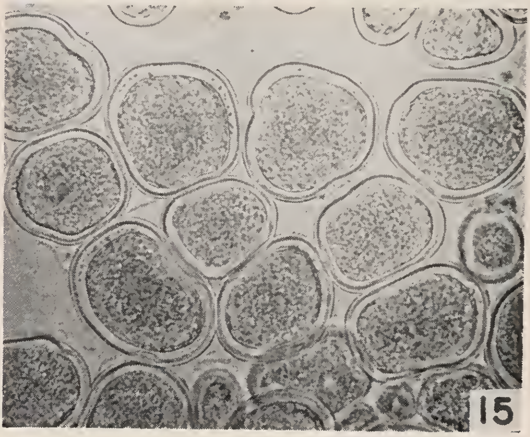
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of endocytes might be analogous to the natural phenomena of macrocyst maturation and germination. However, further experiments failed to substantiate this view, for endocyte reversals did not occur at pH levels that permit growth and other vital activities of the slime mold. Furthermore, in the alternate presence of acid and alkaline solutions an initially endocyte-filled macrocyst could be interconverted repeatedly from an obviously cellular to a seemingly homogeneous state. Such reversal undoubtedly represents a physico-chemical reaction rather than a vital phenomenon, since macrocysts pre-killed with iodine-alcohol or Schaudinn's fixative react as do untreated structures.

The aforementioned tests in combination with certain others have provided substantial information regarding the wall structure of the macrocysts, and specimens treated alternately with alkali and acid and stained with chloroiodide of zinc, followed by Schweitzer's reagent, have proved particularly revealing. The "wall" of a mature, endocyte-filled macrocyst is seen to consist of three strikingly different parts: (1) an outer, loosely fitting primary covering of indeterminate form which characteristically surrounds one or more macrocysts, and may be continuous with, or adherent to, comparable envelopes of adjacent structures (Figs. 24–26); (2) a very much thicker secondary layer that is uniform in thickness, smooth in contour, and usually circular or ellipsoidal in outline (Fig. 26); and (3) an inner membrane formed by the endocytes prior to their disappearance as discrete cellular entities (Fig. 22). Both the primary covering and the secondary wall contain cellulose, apparently embedded within a matrix of more resistant material (Fig. 24–25). Both stain blue in chloroiodide of zinc and both are birefringent when viewed with polarized light, yet neither is completely dissolved by Schweitzer's reagent. However, each loses its birefringence upon the addition of the cuprammonium or an aqueous solution of 72% H_2SO_4. The primary membrane contains relatively little cellulose, embedded within a mucus-like material, and is reminiscent of the slime track and the slime sheath seen in *D. discoideum;* in contrast, cellulose constitutes the principal building substance of the thick secondary wall (Figs. 23 and 29), just as it does in the sorophore sheath of *Dictyostelium* (Raper and Fennell, 1952). The tertiary wall, if it may be so designated, contains no cellulose and represents a continuous film formed in a peripheral position by the mass of differentiating endocytes. This thin, innermost layer is non-rigid and contracts or expands with changes in the volume and character of the protoplasmic material that

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PLATE III

FIGURE 10. Ten-day-old macrocysts produced on 0.1 L–P medium containing Nile blue sulfate, untreated. × 360.

FIGURE 11. The same macrocysts following exposure to 1.0 N NaOH; note that all evidence of the constituent endocytes has disappeared. × 360.

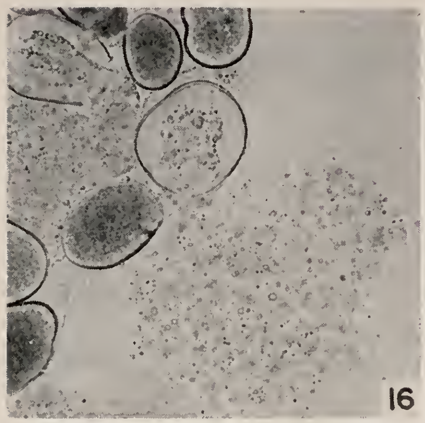
FIGURE 12. The same macrocysts following the application of 1.0 N HCl; note how the endocytes have reappeared. × 360.

FIGURE 13. Six-week-old macrocysts showing the typical contracted homogeneous content of such structures, untreated. × 360.

FIGURE 14. The same macrocysts following exposure to 1.0 N NaOH; note how the contents have swelled. × 360.

FIGURE 15. The same macrocysts, displaced in their relative positions, following the application of acid; note that the cyst contents have contracted somewhat, but that they show no evidence of a cellular structure. × 360.

Plate IV. Behavior of young and aged macrocysts in Schweitzer's reagent.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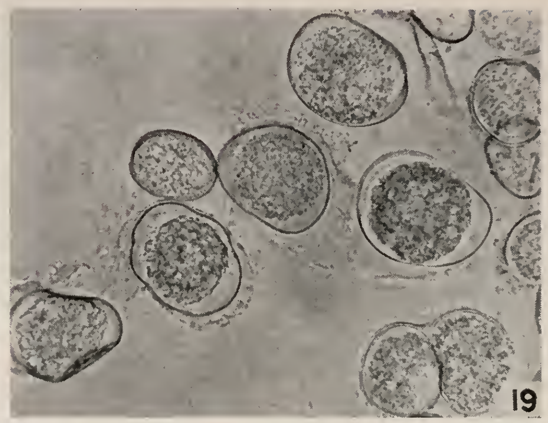
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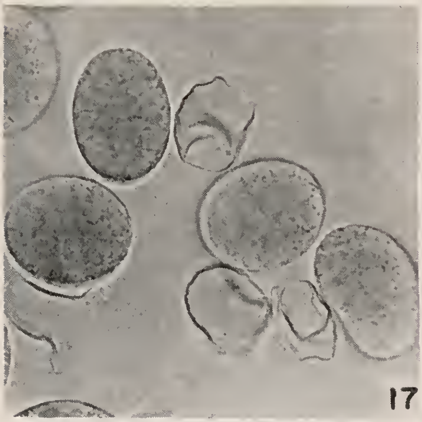
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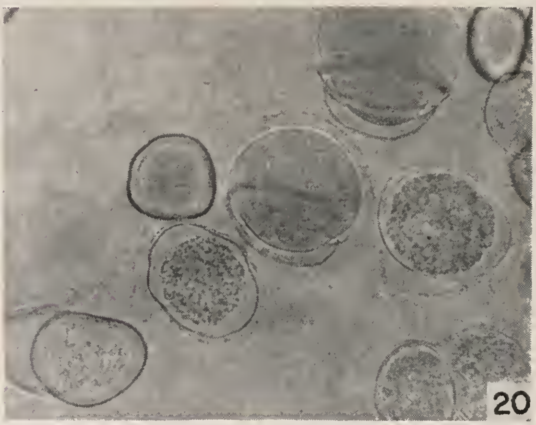
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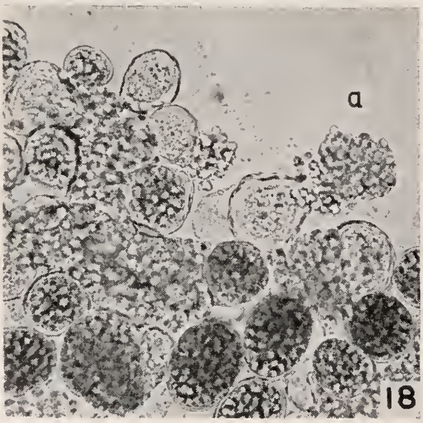

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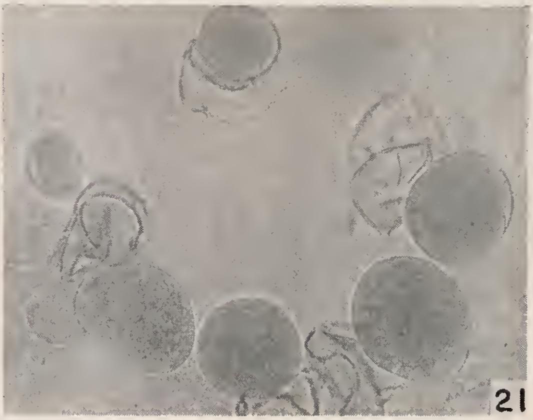
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Figure 16. Young (endocyte-filled) macrocysts following exposure to Schweitzer's reagent, showing how the cuprammonium solution dissolves the cellulose walls of the macrocysts allowing the endocytes, faintly discernible, to escape. × 360.

it envelops, functioning as the tenacious covering that confines the merged cytoplasmic content of the aged macrocyst when this is released by dissolution of the heavy cellulose wall (Fig. 21). In contrast, when an aged but untreated cyst is broken by mechanical pressure this covering obviously ruptures with the cellulose wall which surrounds it (see above).

Similar preparations afforded equally interesting if more perplexing observations concerning the behavior of the contracted acellular protoplasmic masses present in the older macrocysts. When exposed to NaOH the shrunken content lost the brownish color characteristic of aged cysts and swelled to fill completely the heavy macrocyst wall. Upon the substitution of acid, the brownish pigmentation returned in part and the content again contracted, but not to its former dimensions (Figs. 13–15). Treated with Schweitzer's reagent the content swelled once more and erupted from the disintegrating thick cellulose wall as a single, seemingly homogeneous mass. Significantly, no return to a cellular structure has been observed in the content of any older cysts treated with cuprammonium or NaOH solutions upon the addition of acid (Fig. 15). Thus, there is evidence that the endocytes actually disappear, either by fusion or disintegration, at the time the aging macrocyst assumes a homogeneous appearance. But why does the emergent content remain intact following treatment with cuprammonium (Fig. 21) instead of flowing out freely as when the wall of the untreated homogeneous macrocyst is broken? Does the protoplasmic content represent, in fact, a plasmodium-like mass formed by the fusion of endocytes? Possibly so. Does the macrocyst wall actually consist of three layers, the innermost persisting about the freed protoplasmic body, not because of its inherently greater strength but because it is cuprammonium-resistant? Possibly this is true, for upon the application of slight pressure this bounding membrane breaks, permitting the fine granular content to escape whilst the membrane *per se* remains as a delicate, irregularly wrinkled and contracted envelope exhibiting no birefringence. Alternatively, and more plausibly, if aged macrocysts remain viable, as observations indicate, the thin hyaline envelope thus demonstrated may, in the living state, represent only the inconspicuous and functionally modified protoplasmic membrane of the contracted central body itself.

Two lines of evidence point to the presence of such a semipermeable membrane at progressive stages in macrocyst development. When an endocyte-filled macrocyst is exposed to a saturated solution of NaCl or sucrose, the content as a whole appears to become plasmolyzed and to withdraw from the surrounding thick cellulose wall as seen in Figure 22, plainly demonstrating the presence of a continuous

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FIGURE 17. Older macrocysts (but still endocyte-filled) following exposure to this reagent; note how the nearly homogeneous cyst contents remain intact, and how the formerly heavy macrocyst walls are shrunken following the dissolution of their cellulose content. × 360.

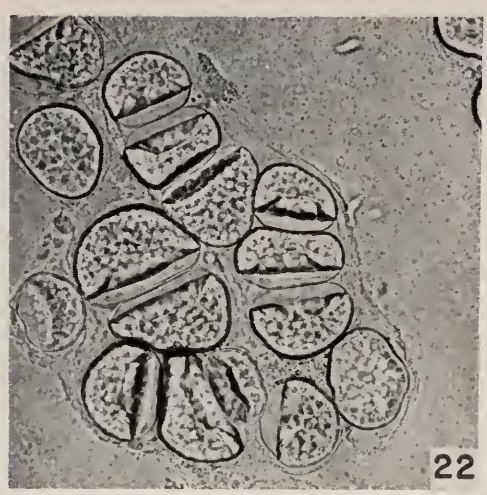
FIGURE 18. Macrocysts of the same age as those shown in Figure 16, treated with Schweitzer's reagent and then exposed to 1.0 N HCl; note how the endocytes have reappeared, even in the freed contents of a macrocyst (a). × 250.

FIGURE 19. Two-month-old macrocysts, untreated. × 360.

FIGURE 20. The same macrocysts following treatment with Schweitzer's reagent; note how the outer cellulose wall is being dissolved and how the membrane-encased content is escaping intact. × 360.

FIGURE 21. The same preparation after an additional 10 minutes, showing the intact cyst contents completely free of the macrocyst walls; the latter are no longer birefringent when viewed with polarized light. × 360.

PLATE V. Macrocyst structure.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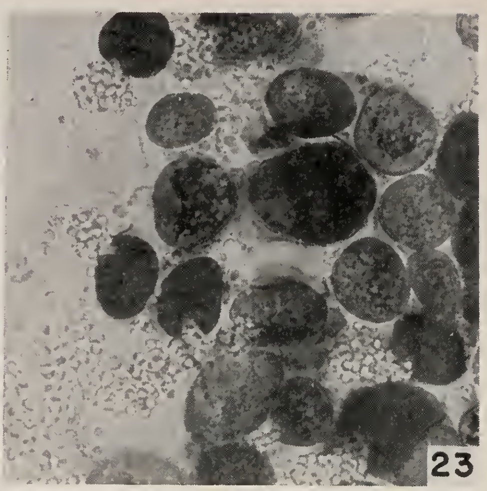
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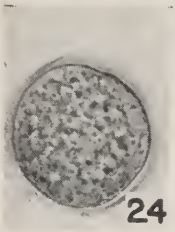
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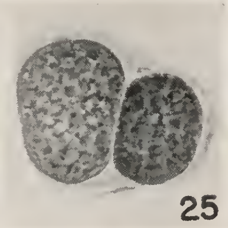
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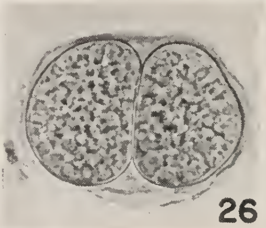
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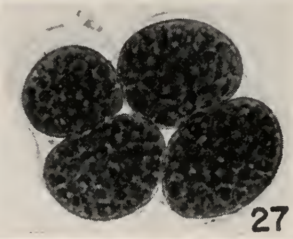
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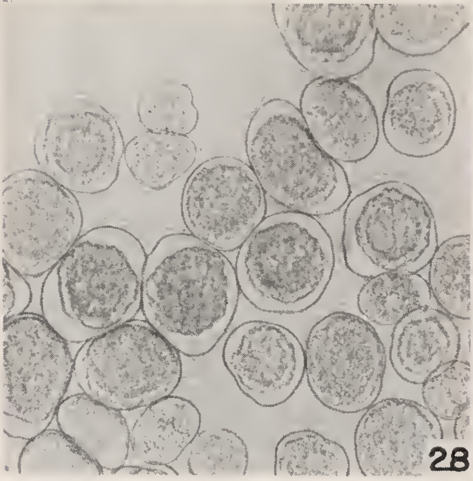
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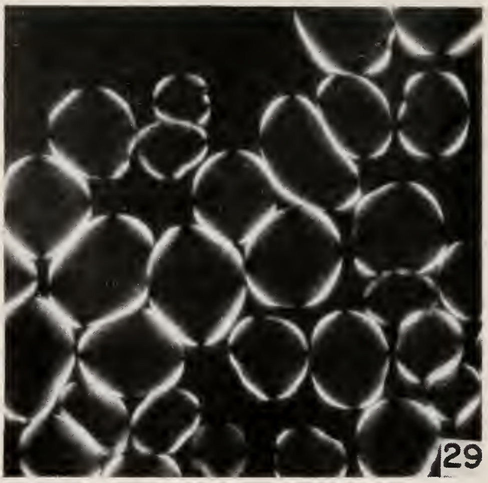
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FIGURE 22. Young (endocyte-filled) macrocysts in the presence of a concentrated sucrose solution; note how a membrane, the "tertiary wall," surrounds the endocytes and in the process of plasmolysis pulls away from the rigid cellulose wall which is external to it. × 360.

FIGURE 23. Macrocysts of comparable age stained with chloroiodide of zinc and then subjected to pressure to release the endocytes. × 300.

and differentially permeable membrane external to the constituent endocytes but internal to the heavy cellulose wall. The same unitary pattern of plasmolysis is seen in macrocysts recently turned homogeneous, and in these there is no suggestion of persistent identity for the contributory myxamoebae or endocytes as one might expect if they remained as indistinguishable but nonetheless discrete cells. Aged macrocysts likewise provide contributory evidence. When placed in relatively large volumes of distilled water such structures show variable response depending upon the temperature of incubation. In preparations held at 10° C. evident swelling of the contracted homogeneous content is observed within 72 hours, and after 10 days many empty macrocyst cases, together with abundant free myxamoebae, may be observed (Figs. 41–42). At 15° C. little swelling occurs, even after 10 days, and only an occasional empty case may be seen. At 20 to 25° C. these responses are almost completely lacking (Fig. 40). This behavior is interpreted to indicate a selective permeability mediated by a low and favorable incubation temperature, and it is most unlikely that this could be attributable to the more conspicuous cellulose wall. The evidence would seem to point, indisputably, to the membrane that surrounds the shrunken cyst content.

The formation of macrocysts is observed not infrequently in *Dictyostelium minutum*, but their occurrence in strains diagnosed as *D. mucoroides* is relatively rare. In fact, not more than a half-dozen such isolates have been encountered among the hundreds of strains of the latter species that we have examined. In view of this, it is surprising and noteworthy that Brefeld (1869), in his description of *D. mucoroides,* reported objects believed to be similar to the macrocysts described above. His cultures were grown on microscope slides, and these structures developed in older preparations of that type. He described the structures as "dwarfed sporangia," since at times there was evidence of a rudimentary stalk in the "spore-forming plasm." He reported this type of sporangium to be enclosed by a comparatively thick membrane of cellulose, which upon examination was observed to be stratified and was stained violet with chloroiodide of zinc. This account agrees well with our observations of macrocysts submitted to various tests. He made no mention of the germination of "spores" from the "dwarfed sporangia." Rudimentary stalks such as Brefeld described and illustrated (Fig. 3) have not been observed during these investigations, and we believe that he may have observed immature macrocysts and interpreted clusters of differentiating endocytes as representing rudimentary stalks. In our experience, endocytes first appear in a localized central position within the developing macrocyst, but occasionally such cells do extend to the periphery along a particular radius before comparable differentiation occurs throughout the macrocyst. However, we must not overlook the possibility that, in his particular isolate cultivated under different conditions, he may have encountered stages truly transitional between macrocysts and well-formed sorocarps comprised of sorophores and sori. Certain evidence points to such a possibility. The basic similarity of the aggregative processes leading to sorocarp

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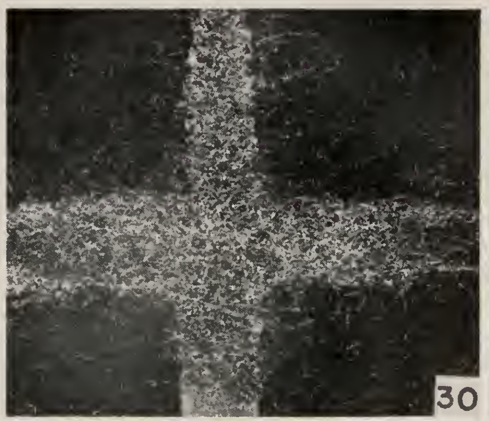
FIGURES 24–27. Selected preparations stained with chloroiodide of zinc to show the loosely fitting primary membranes within which 1, 2 or 4 macrocysts have developed; the bodies shown in Figure 27 are older, hence the heavy macrocyst walls stain darkly. × 360.

FIGURE 28. Two-month-old macrocysts as viewed with normal light. × 275.

FIGURE 29. The same as seen under polarized light. × 275.

Plate VI. Influence of substrates and incubation temperature upon macrocyst formation in *D. mucoroides,* Strain S–28b.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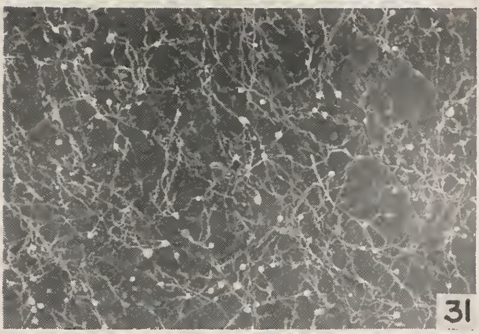
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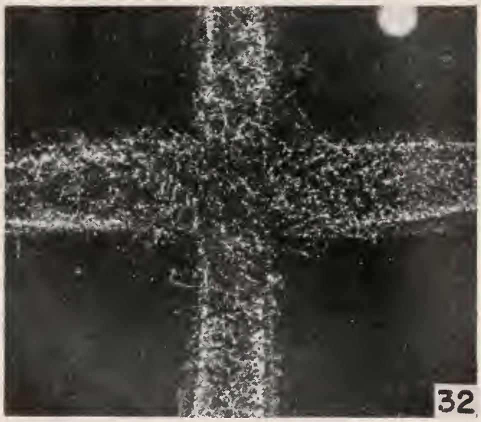
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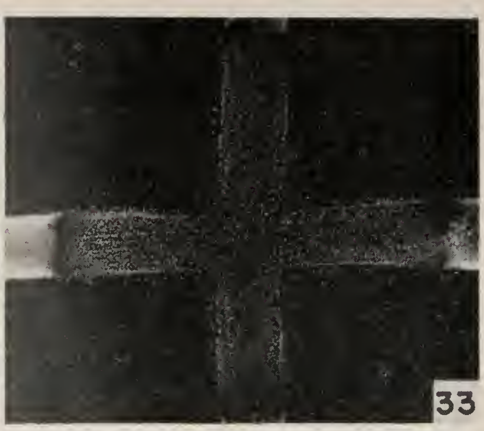
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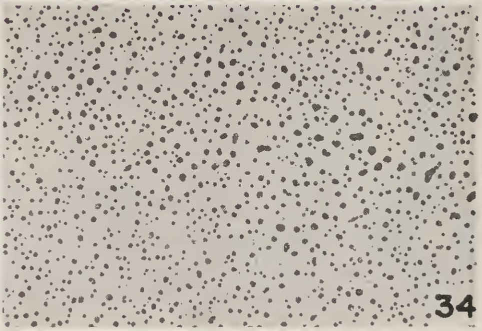
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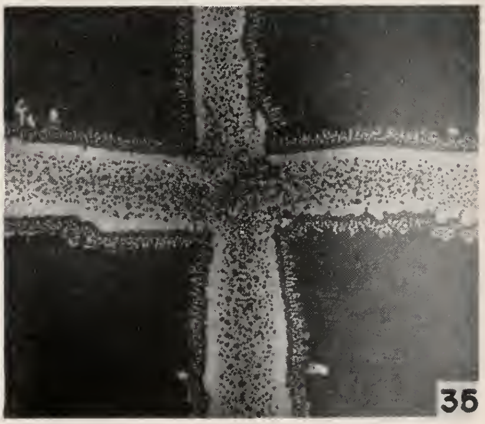
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Figure 30. "Spore-forming clone" grown in association with *E. coli* on 0.1 L–P medium at 20° C. × 2.

Figure 31. Enlarged view of a portion of this culture showing abundant sorocarps and a complete absence of macrocysts. × 5.

Figure 32. The same culture as seen in Figure 30, but growing upon a medium containing 0.1% glucose-0.1% yeast extract, at 20° C. × 2.

formation, on the one hand, and to macrocysts on the other is indeed striking, and we have commonly observed in a single microscopic field separate pseudoplasmodia undergoing differentiation in these two directions simultaneously. Such a situation is illustrated in Figure 36. Unfortunately, we have no adequate explanation of the subtle differences that underlie this contrasting behavior, but it is easily conceivable that one might encounter individual cases where the shift from one to the other of these morphogenetic processes would be incomplete, as suggested by some of Brefeld's illustrations.

The formation of macrocysts may in effect represent an aberration of the normal fruiting process, or it may represent an alternative pattern of differentiation with implications of far-reaching significance that are yet unappreciated. The heavy cellulose wall which it develops bears in many ways a striking resemblance to the sorophore sheath, so essential to the construction of the normal sorocarp (Raper and Fennell, 1952), but with this important distinction: the wall of the macrocyst is secreted external to the whole mass of myxamoebae that contribute to the formation of this body, whereas the sorophore sheath is produced at a critical circular locus within the mass by a limited group of specialized cells which subsequently differentiate as the vacuolate cellular elements of the sorophore itself.

We have, at present, an incomplete picture of the morphology of the macrocyst. We have convincing evidence that they can germinate and re-initiate the life cycle of the slime mold. However, we do not know the fate of the cells which enter the macrocyst, and we do not know the origin of those which subsequently emerge. Until such information is at hand we cannot compare in any definitive sense the morphogenetic processes that underlie these contrasting developmental stages. A thorough cytological and histological study is clearly needed and will be undertaken at the earliest possible opportunity.

Factors influencing the formation of macrocysts

The obvious factor most directly affecting macrocyst formation in *Dictyostelium mucoroides* and *D. minutum* is the inherent genetic constitution of the particular isolate. Within our experience, a minority of *D. minutum* strains and only an occasional isolate of *D. mucoroides* have exhibited this capacity. However, once this ability has been demonstrated for a culture, it is sometimes possible to alter markedly the relative proportions of macrocysts and of normal sorocarps by changing the conditions under which the slime mold is cultivated. The responses of *D. mucoroides,* strain S–28b, have been studied in considerable detail and the observations subsequently recorded apply particularly to that strain. Whereas the various factors that influence the ratios of sorocarps to macrocysts are invariably interrelated, certain conditions which strongly affect these balances have been identified and investigated more or less independently.

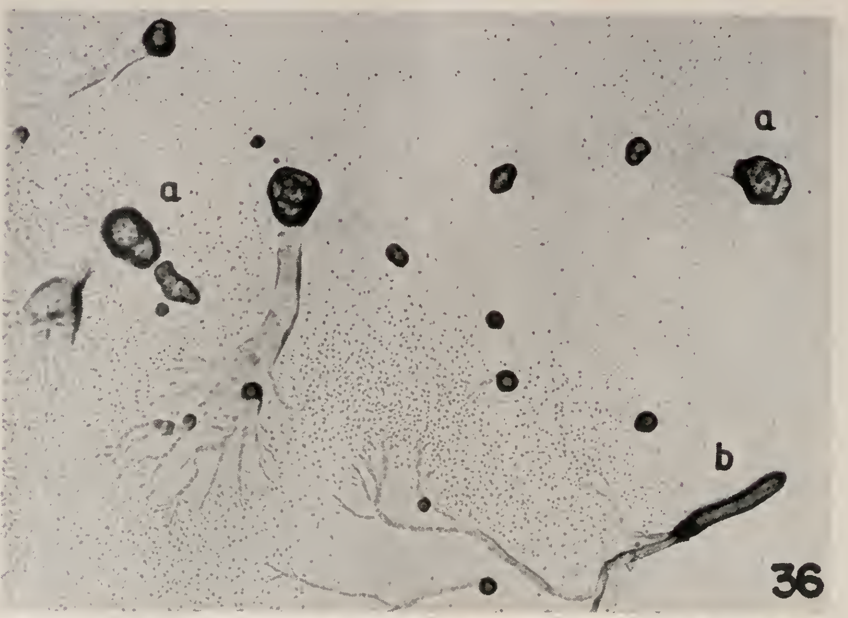
Temperature: Second only to genetic constitution is the influence of the incubation temperature. When cultivated at 24–25° C. in association with *Escherichia*

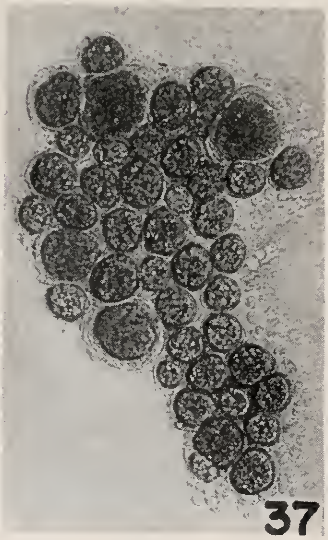
FIGURE 33. "Cyst-forming clone" growing in association with *E. coli* on 0.1 L–P medium at 20° C. × 2.

FIGURE 34. Enlarged view of a portion of this culture showing abundant macrocysts and a complete absence of sorocarps. × 7.5.

FIGURE 35. The same culture as seen in Figure 33, but growing upon a medium containing 0.1% glucose-0.1% yeast extract at 20° C. × 2.

PLATE VII. Macrocyst formation in *Dictyostelium mucoroides,* Strain WS-47.





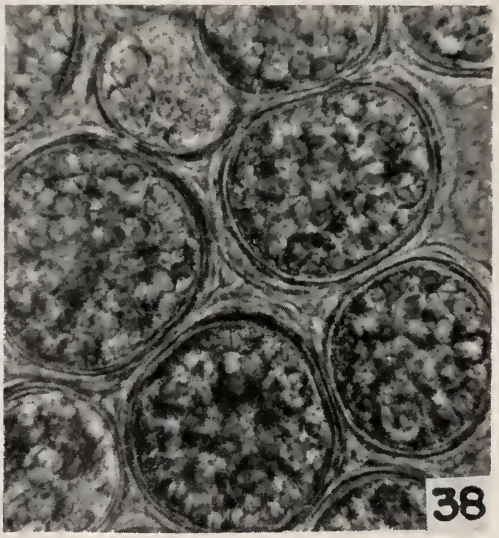
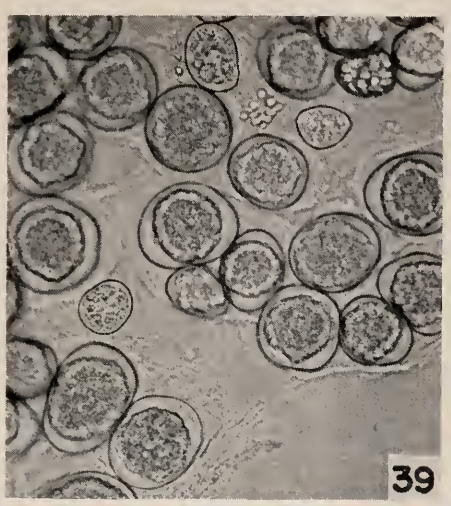


FIGURE 36. Radiate pseudoplasmodia leading to macrocyst formation (a) and to sorocarp formation (b) within the same microscopic field, as seen with low magnification. × 24.

FIGURE 37. Irregular clump of macrocysts developing from a single pseudoplasmodium such as that shown in the preceding figure. × 125.

FIGURE 38. Detail of some of the macrocysts seen in Figure 37. × 600.

PLATE VIII. Macrocyst germination in *Dictyostelium mucoroides*, Strain S–28b.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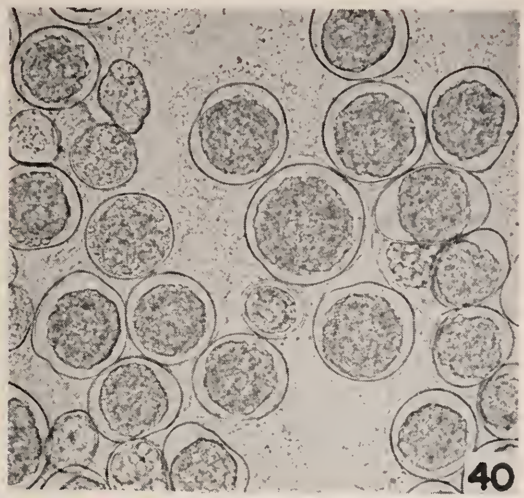
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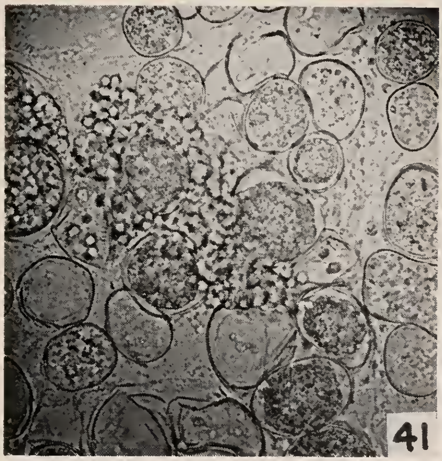
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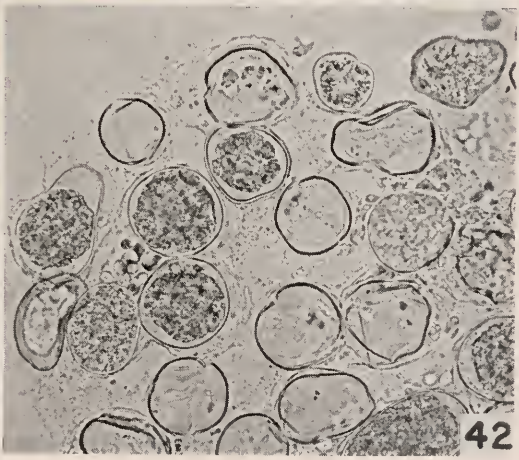
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FIGURE 39. Two-month-old macrocysts incubated continuously at 20° C. and removed from an agar plate immediately before being photographed; note the single macrocyst at upper right which still contains endocytes. × 300.

FIGURE 40. Macrocysts similar to the preceding, but removed from agar and incubated in distilled water at 20° C. for 10 days prior to photography; note that little evident change has taken place. × 300.

FIGURE 41. Macrocysts like the preceding, but incubated for 10 days at 10° C. just prior to being photographed; note the empty cyst cases and the mass of free myxamoebae which have escaped from these. × 300.

FIGURE 42. Photomicrograph showing a second field from the same preparation shown in Figure 41; note the broken walls of the empty cyst cases, also that a few myxamoebae still remain within the germinating cyst at top center. × 300.

coli on 0.1 L–P, hay-infusion or thin hay-infusion agars, the stock strain of S–28b regularly produces both sorocarps and macrocysts in abundance, although individual cultures and even different areas within the same Petri dish commonly exhibit conspicuously disproportionate ratios of these contrasting structures. If the temperature is raised as little as two or three degrees the ratio of macrocysts to sorocarps is increased substantially; conversely, if it is lowered to 20° C. or less this ratio is strongly depressed.

Clonal substrains of S–28b have been isolated which exhibit temperature sensitivity even more dramatically. These were obtained by heating spore suspensions in standard salt solution (Bonner, 1947) for varying lengths of time and then, following appropriate dilution, plating the spores in association with *Escherichia coli* on 0.1 L–P agar. Plaques, evidenced by clearance of the bacteria, developed after four days in plates incubated at 20° C., presumably from single spores. Some of these subsequently developed only macrocysts, others produced only sorocarps. By re-isolation from such contrasting areas, a "cyst-forming clone" and a "spore-forming clone," with strikingly different temperature responses, were isolated (Figs. 30–35). Upon continued recultivation on 0.1 L–P agar, the former characteristically produced only macrocysts at 20° C. but developed abundant sorocarps and scattered macrocysts at 15° C., whereas the latter typically produced only sorocarps at 20° C. but formed both sorocarps and macrocysts at 25° C. Cultures initiated with spores from the cyst-forming clone grown at 15° C. developed macrocysts when recultivated at 20° C., demonstrating a marked degree of genetic specificity in the myxamoebae and spores of the clone. Thus the pattern of cellular differentiation exhibited by different clones of S–28b results from inherited characteristics that are temperature-dependent for their expression.

Culture media: The production of macrocysts is strongly influenced by the substrates upon which the slime mold is cultivated, and these structures are regularly formed in greater abundance upon 0.1 L–P agar than upon media based upon hay-infusion. Their development is even further accentuated if yeast extract is substituted for peptone, this being used either as the sole nutrient or in combination with lactose or glucose. For example, upon 0.1% lactose-yeast extract or 0.1% glucose-yeast extract agars abundant macrocysts are formed by the cyst-forming clone incubated at 15° C. and by the spore-forming clone at 20° C., in each case at temperatures where few or no macrocysts normally develop on 0.1 L–P agar. An intimate association obviously exists between substrate composition and incubation temperatures as these factors affect macrocyst formation, but just how they condition the cultural environment and how they affect the fructifying myxamoebae, influencing them to produce either macrocysts or sorocarps, has not been determined (Figs. 30–35).

Bacterial associate: Dictyostelium mucoroides, S–28b, can be cultivated successfully with a variety of bacterial associates on the nutrient-poor media employed in this investigation. Gram-negative bacteria support better growth of the slime mold than do Gram-positive types, and of the former *Escherichia coli* was the most favorable species investigated. *Aerobacter aerogenes, Pseudomonas fluorescens* and *Serratia marcescens* yielded satisfactory but less luxuriant slime mold growth. The formation of macrocysts and sorocarps followed, in general, the patterns observed with *E. coli.* The myxamoebae of strain S–28b digest or destroy the red pigment (prodigiosin) of *S. marcescens,* hence yield uncolored sorocarps and macrocysts. In marked contrast, the cells of strain NC–12, like those of *D. discoideum*, retain the pigment (Raper, 1937), hence produce sorocarps and macrocysts that are pink in color. *Bacillus subtilis*, a Gram-positive bacterium, supported fair growth of the slime mold and the formation of abundant macrocysts on 0.1% lactose-yeast extract agar at 20° C. *Sarcina lutea*, a second Gram-positive species, permitted only limited growth of myxamoebae and few macrocysts were produced on any substrate. An experiment conducted to determine what effect the age of the nutritive *E. coli* might have on growth and macrocyst formation in *D. mucoroides* failed to reveal any significant differences.

Hydrogen-ion concentration: A heavy suspension of *E. coli* cells previously grown in 0.1 L–P broth and concentrated by centrifugation was cross-streaked on 0.1 L–P agar plates to investigate the effect of pH on slime mold growth and macrocyst formation. The underlying agar substrate was adjusted over a range of pH from 3.3 to 9.2 using the buffers previously cited. In these experiments growth of myxamoebae was obtained between pH 4.5 and 8.0. Slight evidence of aggregation was observed at pH 4.5 but no well-defined pseudoplasmodia developed, and of course no sorocarps or macrocysts were produced. A heavy development of sorocarps and/or macrocysts was obtained between pH 5.5 and 7.0, depending upon the incubation temperature. No growth occurred above pH 8.0 where K_3BO_3 was used as buffer. Questioning whether it was the high pH or the borate buffer which inhibited growth, we made additional tests using KH_2PO_4 as buffer with the addition of NaOH to yield pH levels from 7.5 to 9.0. Growth of the stock culture occurred to pH 9.0, but few and abnormal sorocarps and no macrocysts were formed. Even in the cyst-forming clone incubated at 20° C. where one would normally expect only macrocysts, many sorocarps and very few macrocysts developed on the more alkaline substrates. The pH of the medium did not change appreciably during the period of slime mold growth.

Ammonia concentration: Cohen (1953) reported that ammonia suppressed normal morphogenetic development in the Acrasieae, either inhibiting growth completely, or causing various abnormalities in sorocarp formation. His procedures were carefully followed to ascertain whether different concentrations of ammonia would enhance macrocyst formation. It was thought that if these structures represented aberrant sorocarps, their production might be enhanced under conditions where sorocarp formation was inhibited. However, macrocysts were not formed in any of the ammonia concentrations employed, possibly indicating a suppressive effect comparable to that reported for sorocarps by Cohen.

Relative humidity: The effect of per cent relative humidity on slime mold growth and the development of macrocysts was tested by the procedures previously indicated. Large depression and ordinary flat slides with one ml. of 0.1 L–P agar were employed, rather than Petri dishes, to insure rapid establishment of an equilibrium between the moisture of the agar and the atmosphere in the desiccators. Olive (1902) attributed the encystment of myxamoebae (microcysts) to adverse conditions such as the drying of culture substrates. If macrocyst formation should represent a stage comparable to the encystment of individual myxamoebae, we would have anticipated more macrocysts when the per cent relative humidity was lowered, causing a drying of the substrate. Such did not occur in our tests.

Macrocyst formation in other strains: Attempts were made to enhance macrocyst formation in other strains of *D. mucoroides* (WS–47 and NC–12) and in *D.*

minutum (WS–56–2 and Purdue 8a). Cultural conditions were varied much as previously outlined for *D. mucoroides,* S–28b. However, in these additional slime molds neither the type of culture medium nor the concentration of its ingredients seemed to markedly affect the relative number of macrocysts produced. For example, the proportion of macrocysts to sorocarps remained relatively constant upon media containing yeast extract *vs.* peptone and upon nutrient-rich *vs.* less concentrated media, although total growth of the bacteria and of the slime mold varied substantially with such changes.

A variety of culture media were buffered and adjusted between pH 6.0 and 8.5. All supported growth of the slime mold, and in no case was there an exceptional increase in macrocyst production.

Temperature relationships were examined carefully. *Dictyostelium mucoroides,* WS–47 (Figs. 36–38), produced more macrocysts at 20° and 25° C. than at 15° C., but this response was not so striking as in strain S–28b. A few macrocysts developed in strain NC–12 at 20° C., but at 25° C. only irregular growth of the slime mold occurred, indicating too high an incubation temperature. In *D. minutum,* Purdue 8a, approximately equal macrocyst formation was observed at 20°, 25° and 28° C. In contrast, WS–56–2 formed no macrocysts at 20° C., only a very few at 25° C., and grew very poorly at 28° C.

Germination of macrocysts

The possible role of the macrocysts in the life cycle of *Dictyostelium* engaged our attention from the outset, since it did not seem reasonable that such structures produced in great abundance and under seemingly optimal conditions would represent a terminal and functionless kind of differentiation. For this reason much thought and effort have been given to their germination. In this, as in other phases of the investigation, studies have been centered upon *D. mucoroides,* S–28b, and our researches have been greatly facilitated by the cyst-forming clones. By capitalizing upon their unique temperature responses it has been possible to produce at will large populations of macrocysts under cultural conditions where no spores were formed. At the same time any vegetative myxamoebae which might have adhered to the macrocysts were readily killed by heating at 42° C. for 10 minutes. No growth of the slime mold occurred when the macrocysts were heated at a temperature high enough to kill the mature spores, and such would have been present in any macrocyst preparations taken from the parent culture. The comparative temperature tolerances of the myxamoebae and spores of *D. mucoroides,* S–28b, are shown in Tables I and II, respectively.

Proceeding on the assumption that the macrocysts might represent a resistant stage in the cycle of *Dictyostelium,* they were subjected to a variety of cultural environments and treatments. Plates of 0.1 L–P agar containing abundant macrocysts were alternately frozen at − 10° C. and thawed at + 25° C. in an attempt to instigate germination, but consistent results were not obtained. Growth from macrocysts treated in this way was twice observed after ten days' incubation. Crump (1950) had reported that raising the temperature favored the germination of encysted free-living amoebae, but no germination ensued after heating macrocysts in a water bath for five minutes at 55° C.

At the suggestion of the late Dr. Charles Thom, an attempt was made to stimulate macrocyst germination by spreading a heavy inoculum of pre-grown cysts and *E. coli* on sterilized soil in Petri dishes and incubating the plates at 10°, 15° and 20° C. No evidence of germination was obtained in 28 days, although we had demonstrated previously that growth of the slime mold could take place under these natural conditions.

Brefeld (1869) stated that germination of *Dictyostelium* spores took place only in a nitrogenous medium and suggested fresh horse dung decoction and uric acid media as substrates. Both were investigated. No evidence of macrocyst germination was observed on fresh horse dung agar plates, but growth was obtained, in association with *E. coli,* on 0.05% uric acid agar after eight days incubation at 15° C.

TABLE I

*Temperature tolerance of myxamoebae of Dictyostelium mucoroides, S-28b, suspended in distilled water**

Time in water bath, minutes	Temperature, ° C.	Relative amount of growth per test		
		1	2	3
0	42	++++	++++	++++
5		0	+	+
10		0	0	0
15		0	0	0

++++ = Excellent growth.
+++ = Very good growth.
++ = Good growth.
+ = Limited growth.
0 = No growth.

* Similar results were obtained when myxamoebae were suspended in standard salt solution.

Macrocysts were dismembered in a McShan-Erway tissue homogenizer in the hope that the endocytes thus freed would re-initiate growth under suitable cultural conditions. Such homogenates were mixed with *E. coli* and the resulting suspensions spread on 0.1 L–P agar plates. No growth of the slime mold ensued, but the possibility of serious injury to the endocytes could not be discounted.

Despite the negative results obtained, the seeming logic of this approach led us yet again to attempt the dissolution of the macrocyst wall by other means as a possible aid to germination and regrowth. The procedure employed was probably ineffective *per se,* but in performing the experiment the "treated" macrocysts were incubated at a variety of temperatures which provided a clue to cultural conditions where germination not infrequently occurred. A cellulase preparation, contributed by Dr. Emory G. Simmons, was investigated as a means of digesting the heavy enveloping macrocyst wall. The enzyme was employed as a 1% solution in $M/20$ citrate solution at pH 5.0. Macrocysts were harvested from cultures where no spores had formed, suspended in the cellulase-citrate solution, and heat-treated to kill any adherent myxamoebae. Germination occurred in varying amounts in the cellulase-treated macrocysts and also in controls similarly heat-treated in standard salt solution. The earliest evidence of germination was observed after 6 days' incubation, and new plaques continued to develop for as long as 22 to 40 days at varied temperatures. The percentage germination was approximately the same for macrocysts heat-treated in the cellulase-citrate and in the standard salt solutions. The earliest evidence and the greatest amount of germination were observed among the macrocysts incubated at 15° C., with decreasing amounts to 25° C. Actual germination of a macrocyst, or of the endocytes contained within it, was not then observed, but in some instances one or more empty macrocyst cases were evident where a plaque of growth occurred.

TABLE II

Temperature tolerance of spores of Dictyostelium mucoroides, S-28b, suspended in standard salt solution (Test 1) and in distilled water (Tests 2, 3, and 4)

Time in water bath, minutes	Temperature, ° C.	Relative amount of growth per test			
		1	2	3	4
0	42	++++			
90		++++			
0	50	++++	++++		
65		+	+		
0	55	++++	++++		
5		++	+++		
10		0	+		
15		+	+		
20		0	+		
25		0	0		
30		0	+		
35		0	0		
0	60	++++	++++	++++	++++
5		+++	++	+	++
10		+	++	+	0
15		0	++	0	0
20		0	+	0	+
25		0	+	0	0
30		0	0	0	0
0	65	++++	++++	++++	
5		+	+	0	
10		0	0	0	
15		+	0	0	
20		0	0	0	

++++ = Excellent growth.
+++ = Very good growth.
++ = Good growth.
+ = Limited growth.
0 = No growth.

It was now hoped that even though the percentage viability was apparently low, a few macrocysts might be seen to germinate if these were carefully isolated and observed periodically over a period of several days. Single macrocysts of different ages (3, 15 and 35 days) were selected, heat-treated to kill any vegetative myxamoebae, and placed individually in marked squares on 0.1 L–P agar plates smeared with *E. coli.* Evidence of macrocyst germination was noticed after incubation at 15° C. for five days.

A series of experiments was undertaken to determine whether macrocysts of a particular age would germinate more readily than cysts of other ages; and since presumptive germination had seemed to vary at different incubation temperatures, special consideration was given to this matter. To obtain macrocysts of specific ages, a heavy suspension of myxamoebae of the cyst-forming clone and *E. coli* was spread over the surface of 0.1 L–P plates and incubated at 20° C. for 5, 10, 15, 20, 25 and 35 days. These macrocysts were then harvested, heat-treated to eliminate all vegetative myxamoebae, spread on fresh agar plates with *E. coli,* and incubated at 5°, 10°, 15°, 20° and 25° C. for 5 to 6 weeks. The results of these experiments are summarized in Table III.

TABLE III

Percentage germination among macrocysts of Dictyostelium mucoroides (No. S-28b) of different ages, and the number of days before such germination was observed at different incubation temperatures

Age of macrocysts, days	Temperature of incubation									
	5°		10°		15°		20°		25°	
	Days	%	Days	%	Days	%	Days	%	Days	%
5 (1)*	35	0	35	0	35	0	35	0	35	0
(2)*	44	0	18	0.6	30	0.2	30	0.07	38	0
10 (1)	35	3.4	20	3.1	22	7.2	28	4.5	42	0
(2)	30	+**	14	+	14	3.0	25	0.7	30	0
15 (1)	38	1.6	18	2.9	12	1.2	18	5.5	38	0
(2)	43	0	20	+	20	+	20	+	37	0
20 (1)	25	0.5	18	1.8	12	1.0	12	1.0	32	0.2
(2)	38	0	23	1.0	15	0.9	15	1.0	29	0
25	38	+	15	4.0	15	+	18	1.0	30	0
35	28	+	20	+	8	0.2	8	0.1	20	0.07

* Indicates separate experiments.

** (+) Indicates germination of macrocysts, but percentage of total population could not be calculated.

Substantial growth of the slime mold was obtained in certain of the above tests, particularly in plates inoculated with 10- and 15-day-old macrocysts incubated at intermediate temperatures, the highest percentage (7.2%) being observed in 10-day cysts incubated at 15° C. The prevalence of empty macrocyst cases in the developing plaques, the prior heat-treatment of the cysts to kill adherent myxamoebae, the carefully prepared and examined source plates from which the macrocysts were taken for these experiments, and the observed presence of empty macrocyst cases at central locations within many plaques, all convinced us that the observed growth must have developed from germinated macrocysts.

Nevertheless, we had not actually observed this phenomenon, and even in the most consistent macrocyst-forming culture it is possible that a minute sorocarp could go unobserved and that an occasional spore, which would not be killed by heating to 42° C., might be carried over with the implanted macrocyst. An experiment was carried out to determine whether an occasional spore, if present, might have served as the initiator of the plaques of amoeboid growth in the macrocyst germination plates. It was known that growth from spores would eventually occur at all of the incubation temperatures employed (5° to 25° C.); therefore, the times required for plaques to develop from individual spores under cultural conditions duplicating the above were determined. At 20° C. plaques were evident within 4 days, at 15° and 25° C. within 6 days, at 10° C. within 11 days, while at 5° C. growth was not evident until 25 days. Since plaque formation is usually optimal at 15° C. on the macrocyst germination plates, and does not become evident until after 8 to 11 days, these results provided additional evidence that the observed growth resulted from macrocysts and not from occasional contaminating spores.

Having determined the optimum cyst age and the incubation temperature that are favorable for macrocyst germination, 10-day macrocysts in association with *E. coli* were implanted on freshly poured plates and on sterile Maximov slides containing 1.0 ml. of 0.1 L–P agar to observe the germination of the macrocysts directly. Realization of this objective proved unexpectedly time-consuming, but it was accomplished. Germination in this instance, as in the majority of cases observed up to this time, was from a macrocyst filled with endocytes at the time of implantation in the test culture. Pre-germination changes were not observed but it is assumed that the heavy macrocyst wall was ruptured either by swelling of its content and/or by enzymatic dissolution (see below).

Re-examination of Table III reveals that maximum germination occurred among the 10-day-old macrocysts; *i.e.,* structures which were packed with endocytes at the beginning of the tests. More significantly, appreciable germination was recorded for some of the older macrocysts, notably the 25-day cysts incubated at 10° C. This result is especially interesting since cysts of this age would have already lost their discrete endocytes, in the great majority of cases, and would have assumed the homogenous appearance that characterizes aged cysts. Thus presumptive evidence was obtained that macrocysts of the latter type are capable of germination—presumptive because even in preparations taken from cultures after several weeks occasional macrocysts are seen in which the endocytes remain distinct, and the recorded germination could have resulted from such non-homogeneous structures. The improbability of this explanation was subsequently demonstrated. Blocks of 0.1 L–P agar bearing abundant homogeneous macrocysts aged 6 and 8 weeks were placed in sterile Petri dishes, flooded with sterile distilled water, and incubated at 10°, 15°, 20°, and 25° C. Within only 10 days approximately half of the cysts of both ages incubated at 10° C., and only at this temperature, had germinated among populations where prior examination had established that only occasional cysts (2–4%) were still in the endocyte stage at the beginning of the experiment (Figs. 39–42). An understanding of the intracystic events which transpire during the progression from endocyte differentiation to their subsequent disappearance, and from this through the stage of seeming homogeneity and protoplasmic contraction to the eventual swelling of this mass and the reappearance of amoeboid cells at the time of germination, must await careful cytological investigation. For the present we can only record that in the presence of a proper aqueous environment and at a favorable temperature the previously shrunken "protoplast" (long believed doubtfully viable) swells and gives rise to myxamoebae which escape during cyst germination. Additionally, there is evidence that these myxamoebae, or the parent coenocyte (?), produce cellulolytic enzymes which facilitate rupture of the heavy cyst wall, for viewed with the polarizing microscope the empty cyst cases contain conspicuously less cellulose than do the walls of macrocysts still ungerminated.

Formation and germination of microcysts

Many members of the Acrasieae are characterized by a second, simpler type of encystment stage where individual vegetative myxamoebae round up and become encased by relatively thin protective membranes (Fig. 1). The walls of these resting cells, or microcysts, like those of the macrocysts and the more resistant spores, are predominantly cellulosic in composition. As reported by Olive (1902), there is ample evidence that these form in response to sub-optimal growth conditions. It is probable that the myxamoebae of any member of the Acrasieae may enter such a stage temporarily, but they are most commonly encountered, and in greatest numbers, in isolates of *Dictyostelium minutum, D. polycephalum, Polysphondylium pallidum,* and *Acytostelium leptosomum.*

Microcysts of several species of the Acrasieae were examined to determine their method of germination. Microcysts were placed in hanging-drop slides in thin-hay broth with killed cells of *E. coli* and incubated at 25° C. Within two days many of the microcysts had germinated, as evidenced by the number of free, feeding myxamoebae and by the empty microcyst cases from which these had emerged. Previous workers (Olive, 1902) had not reported true excystment of the microcysts, but had intimated that the myxamoebae absorbed the protective covering, or wall, during germination. The emptied cases are extremely delicate and hyaline, and some reveal a fairly obvious opening at one side through which the myxamoeba escaped. They do not germinate by the emergence of the protoplast through a pore or exit tube, neither do they appear to split as do the spores of most species; rather, germination appears to take place by the dissolution of a fractional portion of the microcyst wall. The cyst cases stain violet-blue with chloroiodide of zinc and show a weak birefringence, indicating that they contain some cellulosic material.

In *Dictyostelium mucoroides* microcysts are about twice the dimensions of the endocytes that comprise the macrocysts, ranging from about 5.0 to 7.5 μ in diameter and being generally spheroidal.

Discussion

Intriguing questions are posed by the macrocysts of *Dictyostelium* with regard to their morphogenesis and their probable primary function in the life-cycle of these slime molds. Do they represent a normal but generally unrevealed stage in the development history of the Acrasieae, *i.e.*, could they be demonstrated in all members of the group if we but knew the conditions required to evoke them? Do they provide a resting stage whereby these micro-organisms survive otherwise impossible environmental conditions? Do they perhaps constitute some unanticipated manifestation of a sexual stage? Or do they represent, as their superficial appearance might suggest, groups of myxamoebae so thwarted in their normal morphogenesis that they become "captives" doomed to a type of terminal differentiation approximating that of sterile stalk cells? Does the identity of the contributory myxamoebae remain unchanged during the formation of the endocytes, and do the latter in some altered form persist to once again emerge during germination as myxamoebae capable of perpetuating the species? For certain of these questions we have succeeded in providing partial answers.

Brefeld illustrated some "dwarfed sporangia" that contained differentiated cells which he interpreted as representing elements of abortive stalks, and early in this investigation we questioned whether the endocytes might not in fact reflect a type of cellular differentiation of this type. More careful examination has established beyond question that such is not the case despite certain superficial similarities in appearance. The walls of the endocytes contain no demonstrable cellulose and the content of such cells is actually condensed, whereas the walls of true stalk cells contain cellulose and the cell content is strongly vacuolate, occupying a peripheral position within the semi-rigid cell. Upon treatment with alkali (*e.g.*, 1.0 N NaOH) the walls of stalk cells do not disappear as do those of the endocytes.

Much evidence supports the belief that macrocysts arise through an orderly and natural morphogenetic process, and hence in no wise represent aberrant fruiting structures. For those strains which produce them, they would appear to be no less normal than the sorocarps which regularly develop under similar or, in some instances, altered conditions. A measure of homology is suggested by the basically similar aggregative process which precedes the formation of both types of structure. The pseudoplasmodia leading to macrocyst formation are generally diminutive, but this condition is not a necessary precedent to their formation. Additionally, the myxamoebae entering a pseudoplasmodium destined to form macrocysts rarely show the marked elongation characteristic of cells entering larger aggregations, but this weak cellular response is believed to indicate degree rather than difference, *i.e.*, to reflect a feeble aggregative stimulus incident to, or responsible for, the small pseudoplasmodium.

A point of similarity should be noted between the morphogenetic processes leading to the formation of gregarious sorocarps in certain species (*e.g., D. minutum* and *D. lacteum*) and to clustered macrocysts in *D. mucoroides,* for in both situations the magnitude of the initial pseudoplasmodium often exceeds the number of myxamoebae that can effectively collaborate in producing a single sorocarp or macrocyst. In the former instance, secondary centers appear soon after the overall pattern of the wheel-like aggregate becomes evident, and from each of these a separate sorocarp subsequently develops; in the latter case, multiple loci of endocyte formation similarly appear within the initial aggregate, and from each of these later develops a discrete and typical macrocyst.

Substantial differences characterize subsequent steps in the two morphogenetic processes, and there is little if any evidence to suggest that the macrocysts represent modified or abortive sorocarps. The latter can be produced in any known species by a variety of devices (*e.g.,* unfavorable pH, increased temperature, etc.), but in no observed instance have such abnormal fruits presented a pattern which is remotely suggestive of macrocyts. Rather, they assume the form of *Guttulina*-like fructifications wherein the myxamoebae produce irregular mounds and undergo incomplete differentiation, but they never form a common protective wall about the mass of cells so assembled.

The heavy cellulosic wall of the macrocyst bears a structural likeness to the sorophore sheath of the normal sorocarp, but, as noted earlier, the relative positions at which these are deposited by the constitutive cells are quite different. Furthermore, the formation of the sorophore sheath is antecedent to cellular differentiation in sorocarp building, whereas it lags behind this phenomenon in macrocyst construction. There is yet another difference which may prove highly significant. Bonner *et al.* (1956) demonstrated that the sorophore sheath is secreted by an ever-changing epithelium-like layer of myxamoebae that are oriented perpendicular to the surface of the wall being deposited, whilst in the macrocysts the last remaining amoeboid cells, hence those adjacent to the developing wall, are oriented in quite the opposite direction. Only in those cultures where seemingly identical and intermixed pseudoplasmodia give rise either to sorocarps or to macrocysts, as in strain WS–47, do we find evidence that the two morphogenetic pathways may be closely allied, and in these we have at present no concept of what major or minor organizational differences may underlie such divergence. Judging from Brefeld's illustrations (1869), it is possible that he may have seen so-called "dwarfed sporangia" that were transitional between sorocarps and macrocysts, but no structures of this type have been observed in our cultures. Finally, we would reiterate that the body of evidence presently available points to macrocyst production as representing an alternative but thoroughly normal morphogenetic pathway that is an inherited character possessed by occasional strains of *D. mucoroides* and by many isolates of *D. minutum.* The isolation of contrasting "cyst-forming" and "spore-forming" clones in strain S–28b strengthens this belief, as does also our inability thus far to induce macrocyst formation in any culture of *D. mucoroides* which did not naturally exhibit this capacity at the time of its isolation.

In contrast to this situation, the capacity to produce microcysts seems to be generally present among the Acrasieae, and it is suspected that every isolate may, under certain variable environmental conditions, exhibit such a resting stage. It should be recognized, however, that this phenomenon is probably totally unrelated to macrocyst formation. Microcysts represent the responses of single myxamoebae to effect a transitory resting stage in the vegetative phase of these slime molds and is perhaps strictly comparable to the encystment of certain small, free-living amoebae. Their natural function is indisputably one of enabling the species to survive otherwise unfavorable environments. The macrocysts, on the other hand, arise through multicellular integration and differentiation and represent the product of a specific morphogenetic process, just as do the sorocarps. This function is still incompletely known.

We have obtained convincing evidence that macrocysts germinate under certain circumstances, emitting amoeboid cells which then re-initiate vegetative growth. But we cannot say with confidence that the macrocysts represent a vital resting stage, as their appearance might suggest. Heat tolerance tests indicate that they can withstand appreciably higher temperatures than vegetative myxamoebae, but they are in turn less resistant than true spores. While the matter has not been explored under conditions that exist in nature, it is possible that they might be produced under circumstances which would preclude the formation of sorocarps and spores, *e.g.*, in strains such as S–28b at elevated temperatures. The general application of this premise is doubtful, however, for many macrocyst-producing strains fail to show a comparable response. Based upon laboratory tests, we could not, at present, conclude that they possess singular survival value.

Possibly they are endowed with other unique properties, a suggestion presently based less upon fact than fancy. We find it difficult to dismiss lightly a structure of multicellular origin which appears to be so highly organized as the older macrocyst. We cannot say with absolute certainty that its content represents a single homogeneous multinucleate protoplast, but such tests as we have applied would seem to support this notion. If the endocytes do actually lose their identity, as appears to be the case, the acellular content of the aged macrocyst would represent a coenocyte, or to use a term more commonly associated with slime molds, perhaps a plasmodium, albeit one that is enclosed within a heavy cellulose wall. Such a plasmodium would of course be quite unlike that which Brefeld (1869) once thought to be present, or that which Skupienski (1920) envisioned as an accompaniment to reported sexuality in *Dictyostelium*. Needless to say, it would represent quite a different structure from the large vegetative body that occupies so conspicuous a place in the life-cycle of the Myogastrales. Clearly, the two could not be regarded as homologous. The same may be said of the plasmodial stages of the Plasmodiophorales, for although their dimensions would be more nearly comparable, these also are never characterized by heavy cellulose walls. Furthermore, no one has yet reported a swimming stage, either gametic or vegetative, for any member of the Acrasieae and such are generally precedent to the formation of plasmodia in each of the other orders.

Here the matter must rest for the present, and definitive information regarding the true nature and ultimate significance of the macrocysts must await further research.

The writers are indebted to Miss Mildred M. Smith for her invaluable aid in the preparation of the illustrations used in this report.

SUMMARY

1. Two encystment stages of cellular slime molds belonging to the genus *Dictyostelium* are described:

The first of these, termed *microcysts*, are unicellular and represent a transient resting stage in the vegetative phase of these simple slime molds. If returned to a favorable environment, microcysts germinated by excystment to re-initiate vegetative growth.

The second encystment stage, termed *macrocysts*, are multicellular and arise through a morphogenetic process possibly alternative to normal sorocarp formation. Myxamoebae aggregate to form typical but generally diminutive pseudoplasmodia which, instead of forming normal sorocarps, subdivide into rounded cell masses that become encased in relatively heavy cellulose walls. Concurrent with this de-

velopment, the myxamoebae that comprise the nascent macrocyst undergo limited differentiation and appear as polyhedral cells with highly refractive membranes. After a period of 10 to 14 days these so-called *endocytes* generally disappear whereupon the content of the macrocyst assumes an acellular, homogeneous appearance. With further aging the protoplasmic content shrinks away from the heavy cellulose wall and in this contracted stage retains its viability for protracted periods. Under favorable conditions of temperature and substrates, macrocysts of different ages germinate to release amoeboid cells which re-initiate the vegetative stage. The sequence of cytological changes underlying this behavior has not been elucidated, and this propagative function may or may not represent the full measure of their significance in the life-cycle of those slime molds which produce them.

2. The ability to produce microcysts is apparently inherent in all members of the Acrasieae, including the genus *Dictyostelium*. In contrast, the capacity to produce macrocysts is more restricted, having been observed only in occasional isolates of *D. mucoroides* and in many strains of *D. minutum*. Various environmental factors influence their production, and from one strain of *D. mucoroides* temperature-dependent "cyst-forming" and "spore-forming" clones have been isolated.

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PHYSIOLOGICAL OBSERVATIONS ON STARVATION AND DESICCATION OF THE SNAIL AUSTRALORBIS GLABRATUS

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It has been shown that planorbid snails, all of which are aquatic pulmonates, can withstand desiccation rather well both in nature and under laboratory conditions (Precht, 1939; Olivier, 1956a, 1956b; Olivier and Barbosa, 1955, 1956). The physiology of desiccating planorbids has, however, received scant attention. Magalhães Neto (1954) observed that five specimens of *Australorbis glabratus* showed a considerably decreased rate of oxygen consumption during desiccation at an unspecified relative humidity. Desiccating aquatic snails retract into their shells; they are unable to feed and hence come under conditions of starvation. Since starvation decreases the rate of oxygen consumption (von Brand, Nolan and Mann, 1948), and since the anatomical relationship of a retracted snail to the source of oxygen is quite different from that of an active one, the following questions arise: Is the reduction in oxygen consumption due mainly to starvation, to difficulties in securing sufficient oxygen, or to desiccation proper? These and related questions are discussed in the present paper.

MATERIAL AND METHODS

Laboratory-reared albino *Australorbis glabratus,* derived from a normally pigmented Venezuelan strain, were used in preference to pigmented specimens because the heart-beat could easily be seen through the shell. This was important, not only because a study of the heart rate under desiccation was interesting in itself, but also in order to establish whether a snail was alive or dead. The usual procedure of placing a desiccated snail in water to observe whether it resumes its normal activities could not be employed because in most of our experiments repeated measurements with the same specimens were required, or because a chemical determination had to be made on desiccated specimens. Most of the snails that appeared dead, as judged by cessation of the heart-beat, were tested further by placing them in water. Of about 200 such snails, only three revived, indicating that our death criterion was reasonably accurate.

All snails initially weighed between 180 and 350 mg. and had fed *ad libitum.* They were freed of excess moisture as described previously (Newton and von Brand, 1955) and weighed to the nearest mg. During starvation, snails (minimum of 28 per series) were kept individually in numbered beakers filled with dechlorinated tap water. They were shifted daily to fresh beakers during the first week of starvation and thereafter twice weekly. During desiccation, snails (minimum of

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[1] Laboratory of Tropical Diseases.

33 per series) were put into individual dry beakers and these were kept at the same temperature (27 ± 1° C.) as the starvation series in desiccators over water or over saturated salt solutions giving a desired relative humidity. The solutions employed gave the following relative humidities, as determined by an Aminco electric hygrometer.

H_2O giving 95 to 97 per cent relative humidity, average 96 per cent
$ZnSO_4$ giving 83 to 87 per cent relative humidity, average 85 per cent
NaCl giving 72 to 76 per cent relative humidity, average 74 per cent
NaBr giving 57 per cent relative humidity, average 57 per cent
$CaCl_2$ giving 28 to 31 per cent relative humidity, average 30 per cent
LiCl giving 13 to 17 per cent relative humidity, average 15 per cent

When survival, weight, heart rate, and rate of oxygen consumption were determined, all snails alive on a given day were used. At the end of the determinations they were returned to the desiccators, or the water-containing beakers, respectively. These snails were used repeatedly until the last specimen died. When chemical determinations were done, only the specimens to be analyzed on a given day were used, while the others remained undisturbed until required for analysis.

The heart rate was determined by counting the heart-beats for one minute under a dissecting microscope.

The conventional Warburg technique was used for the oxygen consumption. The vessels contained 2 ml. of dechlorinated tap water in the starvation series and in the experiments designed to give the pre-desiccation rate. In the desiccation experiments the snails were put into the main compartment of the vessel without water. In these cases the side arms of all vessels, including thermobarometer, contained 0.3 ml. of the same salt solution that was present in the desiccators where the snails had been kept, thus maintaining approximately the same relative humidity. In all cases the temperature was 30° C.

Polysaccharides were determined according to von Brand's (1936) micromodification of Pflüger's method. Total lipids were determined by heating the crushed snail with 30 per cent NaOH in a boiling water bath, acidifying the solution with 7 per cent H_2SO_4, extracting the solution three times with ether, washing the combined ether fractions with distilled water, evaporating the ether, and weighing the lipids on a microbalance after drying at 80° C. For lactic acid [2] and volatile acids the methods of Barker and Summerson (1941) and Bueding (1949) were used, respectively.

All measurements were done on numbered individual snails, with the exception of the volatile acid determinations, where two snails were used. All values are expressed on the basis of the initial, pre-experimental weight of the snails.

RESULTS

1. Survival. Figure 1 shows the survival groups of starving snails at various relative humidities and in water. It is obvious that time of survival decreased with decreasing humidity. When the 50 per cent death times are plotted logarithmically against relative humidity (lower part of Fig. 1), no straight line

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[2] We are indebted to Mr. C. Elwood Claggett for carrying out the lactic acid determinations.

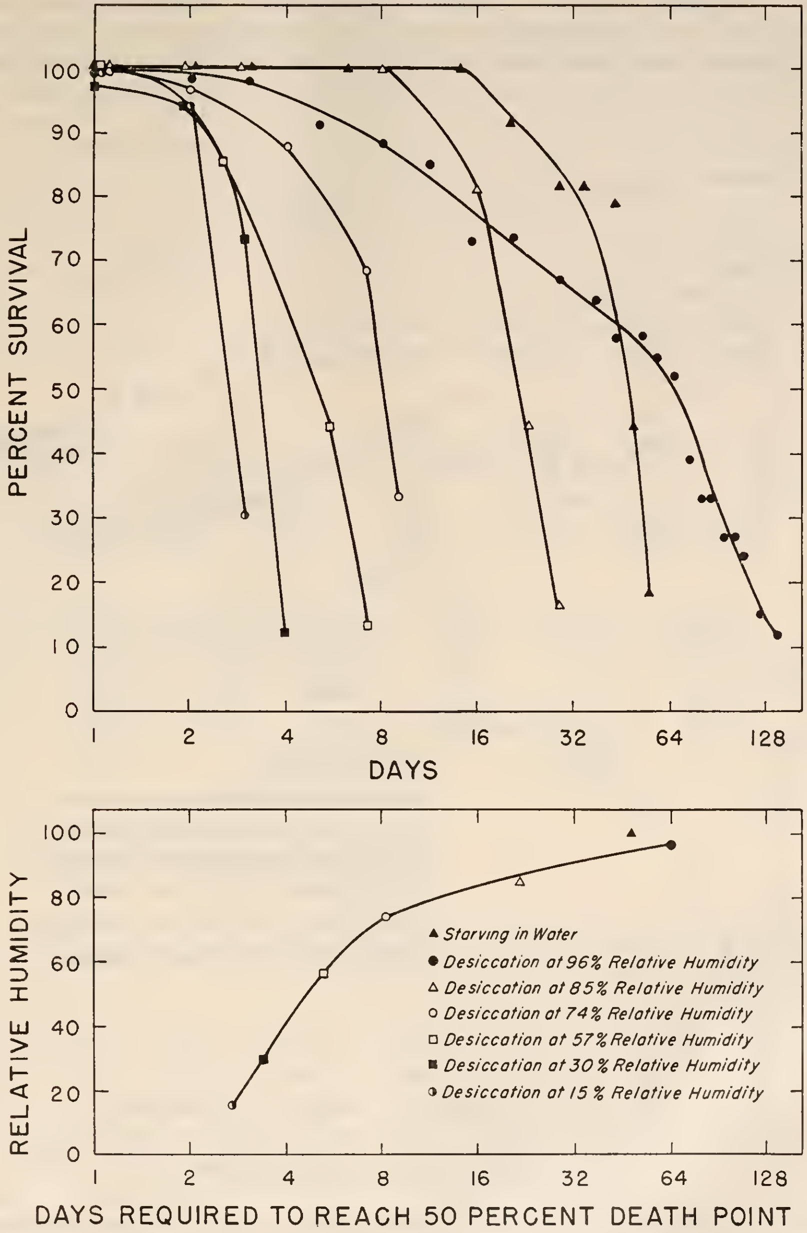

FIGURE 1. Survival of *Australorbis glabratus* starving in water or desiccating at various relative humidities. At the beginning each desiccating series consisted of 33 specimens, the water-starvation series of 28 specimens. The same groups of snails yielded the results shown in Figures 2, 3 and 4.

results. The shape of the curve indicates that no fixed relationship between time of survival and relative humidity exists, but that decreasing humidity leads progressively to an ever more accelerated death rate. Survival during starvation in water was only about half as long as during desiccation over water (96 per cent relative humidity). However, the 50 per cent death points of these two series were closer together. This is due to the fact that the first deaths due solely to starvation occurred later than those from the combined influences of starvation and desiccation.

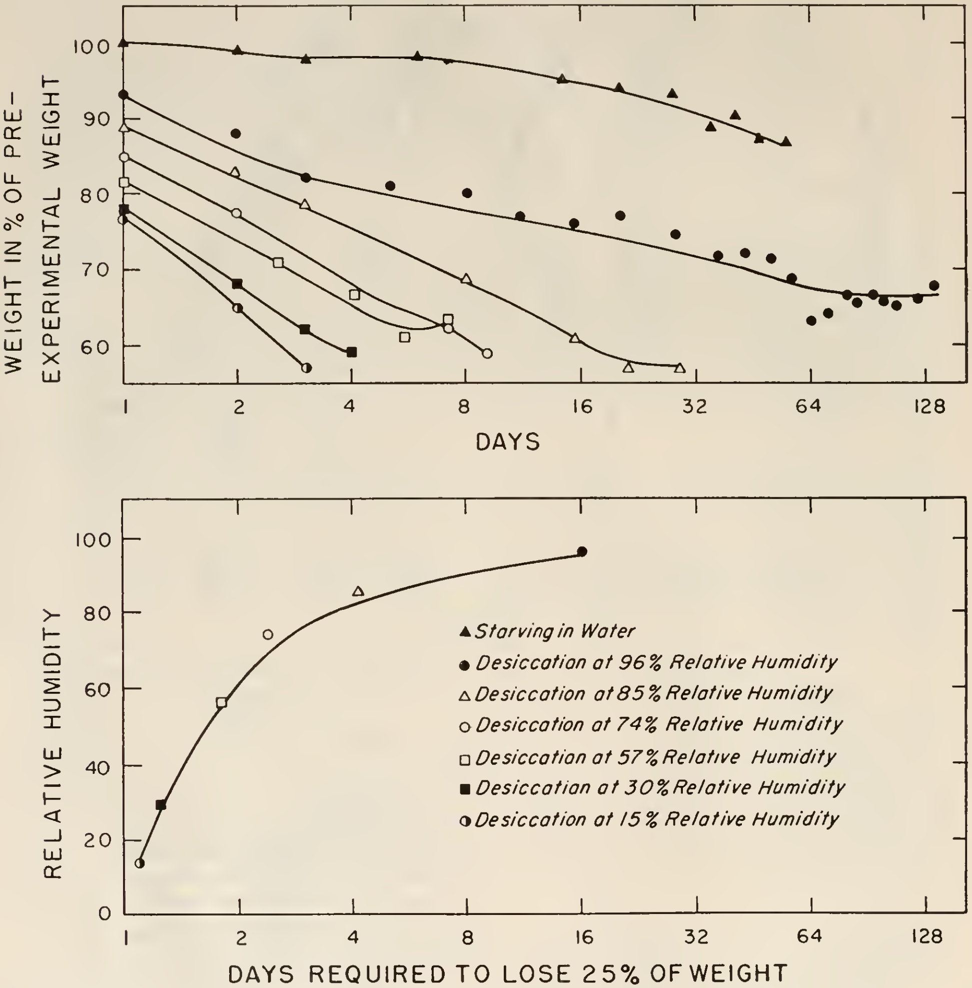

FIGURE 2. Weight relationships of *Australorbis glabratus* starving in water or desiccating at various relative humidities.

2. *Weight loss.* Thirty fed control snails averaged 40.8 per cent dry substance. The shells of 30 other controls, after the soft parts had been removed according to Nolan and von Brand's (1954) procedure averaged 31.6 per cent of the total

weight. The dry weight of the soft tissues was thus 9.2 per cent of the total body weight. Since the shell contains practically no water, the total water found can be ascribed to the soft tissues. Their initial over-all hydration is then calculated as 87 per cent (Table III).

A total weight loss of 13 per cent was observed in snails starving to death in water. It was hence greater than the total dry weight of the soft tissues initially present and it must be concluded that it was due in part to a loss of water, perhaps corresponding to the hydration water of the metabolized organic material.

The weight loss of desiccating snails (Fig. 2) was much more pronounced than that of snails starving in water and was clearly dependent on the relative humidity. The lower part of Figure 2 indicates that the relationship between humidity and weight loss is very similar to that described above for survival. Snails desiccating and starving at 96 per cent relative humidity metabolize during 128 days approximately 50 to 60 per cent of their organic material (corresponding to 4–5 per cent of the pre-experimental live body weight, see Discussion). Since in the humidity range of 15 to 85 per cent practically all snails had died by day 20, their loss of organic material must have been much smaller, and no appreciable error can be introduced if the entire weight loss is here ascribed to loss of water. At these humidities the weight had declined terminally to 60 per cent of the initial, corresponding to a loss of 40/59.2 = approximately 70 per cent of the initial water.

TABLE I

Desiccation of Australorbis glabratus at 85 per cent relative humidity. The figures are per cent of the pre-desiccation values; the figure following the ± sign is the standard error of the mean

Days of desiccation	Heart rate		Weight		Rate of O_2 consumption		Per cent survival
	A	B	A	B	A	B	
1	98±2.6	98±4.2	89±0.5	90±0.7	49±2.6	47±4.5	100
2	100±4.2	107±5.2	83±0.3	85±1.7	46±2.2	47±4.4	100
3	95±3.0	93±4.8	79±0.7	81±0.9	33±2.7	30±3.0	100
8	105±3.7	98±3.7	69±1.0	71±1.1	27±2.1	20±2.4	100
15	93±4.6	95±3.7	61±1.1	64±1.7	28±2.5	30±3.6	81
22	81±9.9	81±9.9	57±1.6	57±1.6	10±2.0	10±2.0	44
28	81±2.4		57±3.7		19±5.0		16

The initial number of snails was 33.
A = Values of all snails alive at specified day.
B = Values of all snails surviving on day 22.

At 96 per cent humidity, on the other hand, survival was much longer and the weight at death was higher, amounting to 65 to 70 per cent of the initial weight. As mentioned above, about 4 to 5 per cent weight loss must in this case be ascribed to metabolized organic material. It is therefore clear that at this high humidity the last animals died before being desiccated to quite the same degree as in the other desiccation series. It is probable that in this case starvation was a contributing factor to death.

It should be realized that the figures summarized in Figure 2 (the following applies also to the data presented in Figs. 3 and 4) are averages for all snails

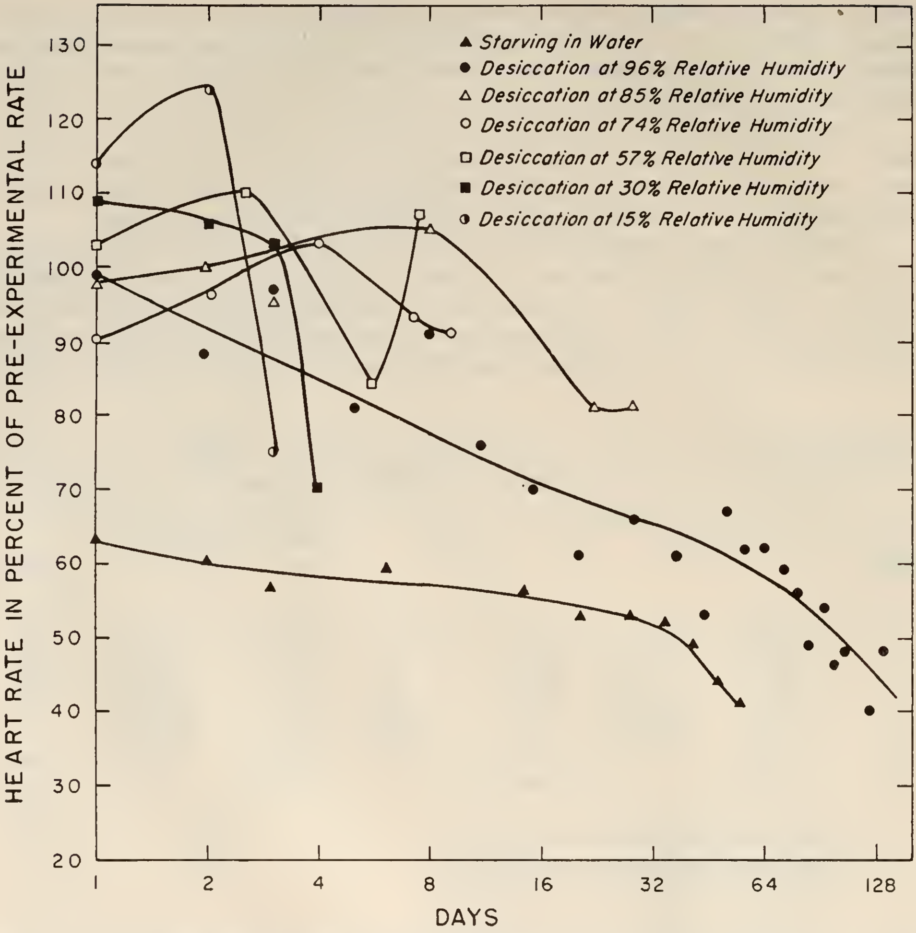

FIGURE 3. Heart rates of *Australorbis glabratus* starving in water or desiccating at various relative humidities.

alive at a specified day. They therefore represent the average changes occurring in a population. Some irregularities in the curves, especially noticeable towards the end of an experiment, are due to the summation of experimental errors and the slightly variable behavior of the individual snails. The curves do not change materially, however, if the data are restricted to snails which are still alive on a day nearing the end of a given experiment, provided the number is sufficient to give a valid average. This is illustrated for one of our series in Table I for weight and other criteria studied.

3. Heart rate. The heart rate of snails (Fig. 3) starving in water slowed precipitously to 63 per cent of the original rate during the first 24 hours of starvation. During the remainder of the starvation period the heart rate declined slowly further, the final value being about 40 per cent of the initial one. In snails desiccating at 96 per cent relative humidity there was no decline during the first 24 hours, but thereafter the heart rate became progressively slower, reaching about the same end-point as in snails starving in water.

A different situation prevailed in snails desiccating at all lower humidities. Within the first few days, there was a period when the heart-beat increased in frequency above the pre-experimental value, this period being followed by one of more or less precipitous decline. While the heart-beat was generally full and regular in snails in water and in air at 96 per cent relative humidity, many irregularities were observed at lower humidities, such as partial contractions of the heart, or

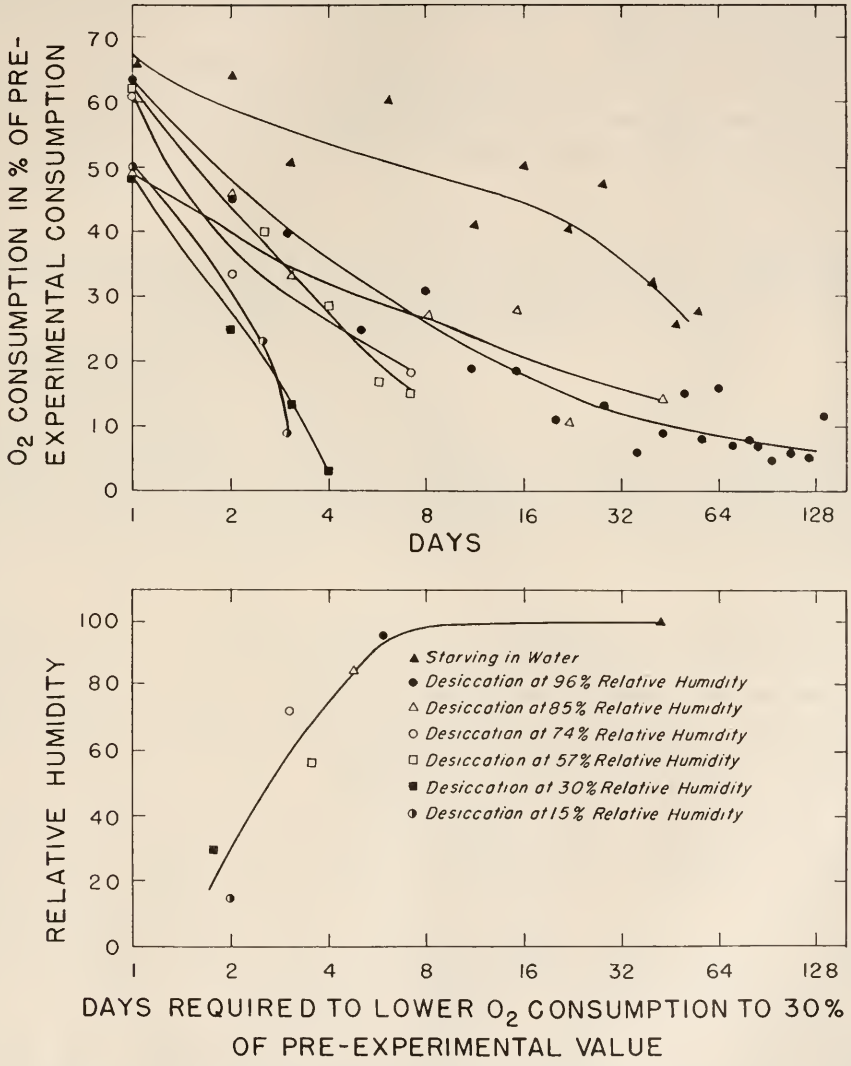

FIGURE 4. Oxygen consumption of *Australorbis glabratus* starving in water or desiccating at various relative humidities.

cessation of the pulsations for a few seconds followed by a period of very rapid contractions. It should be noted that at 57 per cent humidity the last value raises the curve (Fig. 3.) This artifact is due to abnormally high rates in all three surviving snails.

Heart rate apparently had no direct relation to survival under our conditions. For instance, in our series of 33 snails desiccating at 96 per cent relative humidity, 7 snails with initial heart rates of 33 to 37 beats per minute survived an average of 48 days. The other extreme was represented by 5 snails with initial rates of 50 to 63 beats/minute and an average survival of 80 days. After 128 days of desiccation, 5 snails still survived; their average initial rate was 42 beats/minute with 37 and 53 as extremes.

4. *Oxygen consumption.* The rate of oxygen consumption (Fig. 4) declined in all series more or less rapidly, but the decline was slower in the water-starvation than in the desiccation series. The daily variations were more pronounced in the former than in the latter, possibly due to the motility of the snails starving in water as contrasted with the immobility of the desiccating specimens. In the starvation series the final rate was about 30 per cent of the pre-experimental one, while in the desiccation series the endpoint varied from about 20 to well below 10 per cent of the initial value. The rapidity of decline (lower half of Fig. 4) showed a rough correlation with degree of humidity, but it was not so close as that shown between humidity and survival or humidity and weight loss.

TABLE II

Chemical determinations on Australorbis glabratus starving in water or desiccating at 96 per cent relative humidity. All values have been calculated on the basis of the pre-starvation or pre-desiccation live weight of the snails. The figure following the ± sign is the standard error of the mean, the figure in parenthesis indicates the number of determinations

Days	Per cent lipids		Per cent polysaccharides		μ Lactic acid	μ Volatile acid*
	Desiccation	Starvation	Desiccation	Starvation	Desiccation	Desiccation
0	0.76±0.023 (24)	0.65±0.036 (24)	1.03±0.10 (23)	1.29±0.16 (22)	140±27.8 (12)	53±28.1 (6)
10	0.58±0.019 (24)	0.43±0.026 (24)	0.77±0.07 (22)	1.04±0.18 (24)	117±28.1 (10)	11± 5.4 (6)
20	0.54±0.016 (20)	0.38±0.011 (19)	0.69±0.07 (24)	0.69±0.14 (21)	35± 7.9 (12)	18±12.8 (6)
30	0.52±0.019 (19)	0.36±0.011 (19)	0.56±0.08 (20)	0.59±0.12 (22)	0.0 (11)	23± 3.9 (3)

* The volatile acids are expressed as acetic acid, since this is the predominant volatile fatty acid (Mehlman and von Brand, 1951).

A rough inverse correlation probably exists between survival and initial rate of oxygen consumption. Taking the series of snails desiccating at 96% relative humidity, as example, six snails had initial rates varying between 205 and 277 mm.^{3} O$_2$/gm./ hr. with an average survival of 51 days. In four snails the rate varied initially between 82 and 108 mm.^{3} O$_2$/gm./hr. and their average survival was 103 days. The five snails surviving 128 days desiccation had an initial rate of 143 mm.^{3} O$_2$/gm./hr., with 106 and 171 mm.^{3} as extremes.

5. *Chemical determinations.* Chemical determinations were performed only during the first 30 days on animals starving in water and desiccating at 96 per cent relative humidity since snails desiccating at lower humidities died too early. In view of the variability in storage of reserve substances, it was essential to limit

periods of exposure to experimental conditions to those tolerated by all or at least the great majority of specimens employed, since otherwise a possible differential death rate between snails with high and low initial reserves would make valid conclusions impossible.

The data summarized in Table II show that starving and desiccating snails use appreciable amounts of both polysaccharide and lipids, the consumption being more pronounced during the first 10 days than after prolonged exposure to experimental conditions. Snails starving in water used little more of these reserve substances than did the desiccating specimens. In desiccating snails the lactic acid initially present in the tissues disappeared completely within 30 days, while the volatile acid content decreased only slightly.

Discussion

The laboratory strain of *Australorbis glabratus* used in the present studies withstood desiccation fairly well. As was expected, the snails retracted into their shells. They were not capable of forming a true epiphragm which in many other species is an efficient mechanism for preventing excessive loss of water, nor did they produce complete mucus membranes across the shell aperture, an auxiliary mechanism frequently employed (Gebhardt-Dunkel, 1953). Partial mucus membranes were observed occasionally, but they did not seem to change the rate of evaporation materially.

Marked reduction in the rate of oxygen consumption with time was characteristic at all humidities studied and a loose inverse correlation with humidity existed. This reduction was not due solely, and in the series at low humidities not even primarily, to starvation. Snails starving in water maintained a higher rate of oxygen consumption than the desiccating specimens; they must therefore have used their reserve substances at a faster rate. It would then seem that the amount of reserve substances available to the desiccating animals would have sufficed to maintain an equal rate of oxygen consumption if starvation alone were involved. An altered anatomical relationship to the source of oxygen can also be eliminated as the cause of this reduction. If difficulties in securing oxygen played a significant role, a partial shift to anaerobiosis would have been expected. It should be noted in this connection that a partial shift to anaerobiosis can readily be induced in *Australorbis* by exposure to low concentrations of pentachlorophenol (Weinbach and Nolan, 1956) and that a considerable increase in lactic acid content has been reported from aestivating *Pila* (Meenakshi, 1956). In our desiccating specimens, on the contrary, the lactic acid present initially disappeared completely and the volatile acids diminished. There is little doubt that *Australorbis,* at least during desiccation at high humidity, maintained a purely aerobic metabolism despite the deep retraction into the shell.

The lung of a contracted snail is probably largely compressed and it is problematical whether it plays a large role in the gaseous exchanges. Diffusion through the tissues exposed to the air within the shell may have been sufficient. It should be kept in mind that conditions are quite different when a snail retracts into its shell in water. During desiccation the tissues are in direct contact with atmospheric air where the absolute amounts of oxygen are much higher than in water and where diffusion is incomparably more rapid than if the whorl is filled with water. It is therefore most likely that the reduction in oxygen consumption was largely a consequence of desiccation proper, although in the longer-lasting series starvation may well have been a contributing factor.

That loss of water *per se* influences snails can be deduced also from our heartbeat observations. Starvation in water led to a reduction in rate, and a similar reduction, though slower to appear, was evident in snails desiccating at 96 per cent relative humidity. At lower humidities, on the contrary, the period of decline was preceded by one of increased rate and many irregularities in heart action were observed. While the final water loss was not very different, it was more gradual in the 96 per cent series and a difference in over-all tissue hydration was probably present even towards the end of the experiments (see below). On the whole, the impression was gained that at 96 per cent humidity the heart had an opportunity to adapt itself to changed conditions, while this did not occur during the shorter periods involved at lower humidities. It is probable that at the lower humidities increased concentration of organic and inorganic materials accumulating in the blood may have put a strain on the heart. It was not directly demonstrated because *Australorbis* is for technical reasons not suitable for such experiments, but Arvanitaki and Cardot (1932) had found previously a salt concentration of 0.080 N in *Helix pisana* collected immediately after a rain and 0.147 N seven days afterwards.[3]

In humidities of 85 per cent and below, the last snails died when they had lost about 70 per cent of their original water.[4] While this loss is very large,[5] the over-all tissue hydration does not decline to the same extent, because the remaining water hydrates the tissues of an animal whose weight has declined. The general relations between over-all tissue hydration and total water loss are shown in Figure 5. This figure is drawn on the assumption that the organic material remains unchanged, and is therefore valid only in cases of very rapid desiccation. In experiments of long duration, such as at 96 per cent relative humidity, a considerable percentage of tissue is lost. While no exact data could be secured, a final loss of 50 to 60 per cent appears possible (see below). If this loss is taken into account, the over-all tissue hydration was about the same at the beginning as at the end of the desiccation period (Table III). Even if this should be literally true (and no such claim is made), the physiological state of the desiccating snail probably differs from that of snails kept in water. For instance, any loss of water, whether accompanied by a loss of tissue or not, should result in an increased percentage of inorganic material

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[3] It is probably unwarranted, however, to generalize: Pusswald (1948) reported that the blood of the slugs *Arion* and *Limax* lost 84.5 and 92.0 per cent of their initial water, respectively, when the water loss of the entire body was 60 per cent. In *Limax* the percentage of water content of the blood had at this point declined from 97.6 per cent to 77.3 per cent. In snails with external shells the water loss of the blood seems to be less pronounced. Gebhardt-Dunkel (1953) studied five species of terrestrial snails and found a decline in the water content of the blood from initial values ranging in the various species from 97.7 to 98.1 per cent to final values varying between 88.4 and 88.8 per cent shortly before death from desiccation.

[4] Actually, the water loss is probably slightly higher since the water resulting from the oxidation of food reserves has not been taken into account in this calculation.

[5] This resistance to loss of water is not unique. Roots (1956) states that the earthworms *Allolobophora chlorotica* and *Lumbricus terrestris* survive losses of body water of 75 and 70 per cent, respectively. Other invertebrates are more sensitive. According to Biancamaria (1955), the crayfish *Potamon edulis* dies after having lost 15 to 23 per cent of the original water. For older data on resistance to desiccation, see Hall (1922).

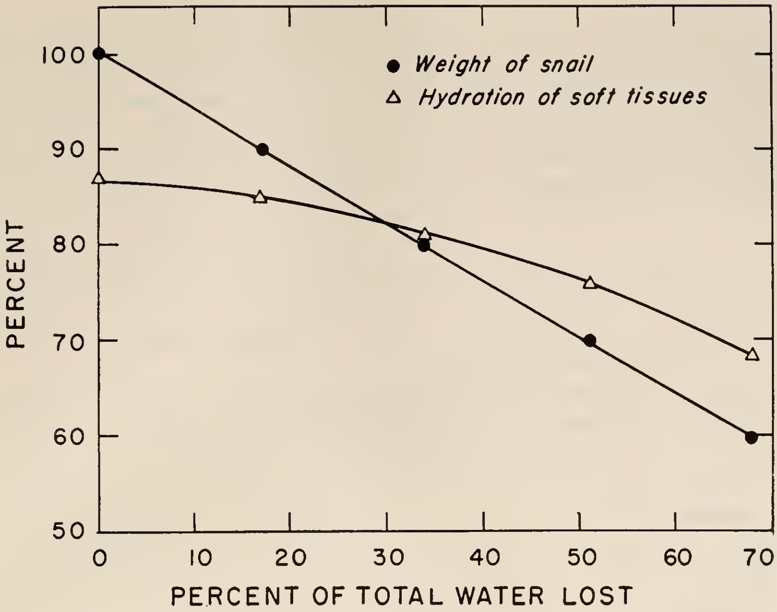

FIGURE 5. Theoretical relation between weight (water loss) and over-all hydration of soft tissues in desiccating *Australorbis glabratus*. Initial water content 87 per cent.

in the remaining tissues, unless the snail is capable of incorporating the excess into the shell, a point that was not studied. Computations from data presented by Buck and Keister (1949, Fig. 7) show that in flies, also, considerable water loss may occur without decrease in over-all tissue hydration. This suggests that the phenomenon may be widespread.

The endogenous foodstuffs during desiccation were studied only at 96 per cent relative humidity where a consumption of polysaccharides, lipids, lactic and volatile acids was found. Since there was no indication of a partial shift to anaerobiosis, total oxidation can be assumed and the oxygen required for it can be calculated. It is also possible to calculate approximately the total oxygen consumed during the desiccation period by graphic integration of the rates determined at the intervals shown in Figure 4. As Figure 6 indicates, the above substances account only for

TABLE III

Calculated over-all hydration of the soft tissues of Australorbis glabratus when 50 per cent of the soft tissues disappear during desiccation at 96 per cent relative humidity and the final total weight of the desiccated snail is 67 per cent of the pre-desiccation value, as was found

	Total weight, mg.	Shell weight, mg.	Weight of soft tissues, mg.	Weight of water, mg.	Per cent water in complex soft tissues+water
Pre-desiccation	100	31.6	9.2	59.2	87
Post-desiccation	67	31.6	4.6	30.8	87

a relatively small fraction of the total oxygen consumption, both during desiccation and during starvation in water. In conformity with other starving organisms, it may be assumed that proteins were the main substrate. A calculation of the total oxygen consumed by snails desiccating at 96 per cent relative humidity for 128 days gives approximately 56 ml. oxygen per one gram original weight. About 9 ml. are accounted for by the oxidation of polysaccharides and lipids during the first 30 days (the additional amount for lactic and volatile acids is negligible). Since these reserves were largely depleted at the end of this period, 47 ml. of oxygen can tentatively be linked with protein consumption, permitting the oxidation of approximately 48 mg. of protein. Since a one-gram snail (initial weight) contains, on an average, 92 mg. of dried soft tissue, the calculated tissue loss would be roughly 50 to 60 per cent. This figure appears possible, since even higher organisms can lose more than 50 per cent of their weight during starvation, *e.g.*, a dog discussed by Pütter (1911) decreased in weight from 19.65 kg. to 9.17 kg., yet recovered upon feeding. Other examples are planarians, which decrease so markedly in size during starvation that their weight loss must be far larger than 50 per cent (Stoppenbrinck, 1905; Berninger, 1911).

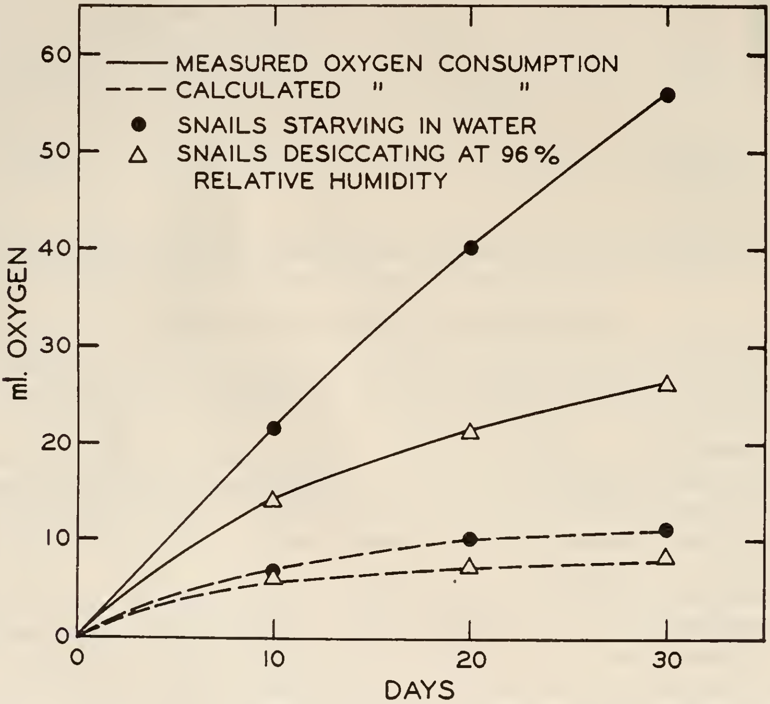

Figure 6. Comparison of oxygen consumption calculated from polysaccharide and lipid consumption, assuming total oxidation, and measured oxygen consumption (total oxygen consumption obtained by graphic evaluation of determinations done at specified intervals; see text). The values have been calculated for one gram pre-starvation or pre-desiccation weight.

SUMMARY

1. Decreasing humidity leads to a progressively more rapid decline of survival time, body weight and rate of oxygen consumption. Snails starving in air of high humidity survive longer than snails starving in water, but their final weight is lower.

2. The heart rate of snails starving in water or desiccating at 96 per cent relative humidity decreases. At all lower humidities a transitory phase of increased heart rate and many irregularities in heart action occurs.

3. During starvation in water and during desiccation, polysaccharide and lipid stores become depleted. Lactic acid disappears completely from the tissues during desiccation and volatile acids diminish.

4. It is concluded that the decrease in oxygen consumption is largely due to desiccation proper but that at high humidity starvation is a contributing factor.

5. Snails desiccating at high humidity have a purely aerobic metabolism. The relationship between the oxygen required for oxidation of polysaccharides and lipids and the total oxygen consumed indicates that protein may be the main substrate during prolonged periods of starvation in water or of desiccation.

6. The percentage of total body water lost and the percentage of water in the tissues do not decrease at the same rate during desiccation, tissue hydration declining at a slower rate. If marked tissue losses occur during long periods of desiccation, the over-all tissue hydration may remain unchanged even if the total water loss is very pronounced.

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SIMILARITIES BETWEEN DAILY FLUCTUATIONS IN BACKGROUND RADIATION AND O_2–CONSUMPTION IN THE LIVING ORGANISM[1, 2]

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Recent studies on fluctuations in O_2-consumption and in spontaneous activity in conditions constant with respect to all factors known to influence organisms, have provided strong evidence that some external fluctuating physical factors are still exerting an influence on protoplasmic systems. The studies were made in conjunction with an analysis of temperature-independent, solar-day and lunar-day, cycles under constant conditions. Solar-day cycles have been known for a number of years to be widespread among organisms, and more recently it has become evident that lunar-day cycles also occur.

The evidence for an influence of an external factor has come from the recent rediscovery (see Stewart, 1898, for early literature) of correlations of organismic activities with barometric pressure and its changes (Brown, Freeland and Ralph, 1955; Brown, Webb, Bennett and Sandeen, 1955; Brown, Bennett, Webb and Ralph, 1956). These correlations have recently also been shown to occur in two lag-lead relationships. One is between the *rates* of barometric pressure change at certain specific times of day *n*, *n*-1, and *n*-2 as correlated with biological activity at an approximately corresponding time on day *n*. A second correlation is between the organismic activity at a particular time of day, expressed either in absolute terms or as deviation from the daily mean and the mean daily barometric pressure of the second day thereafter. That these correlations are in no manner responses to pressure changes themselves is clear not only from the lead-correlation of the organism on barometric pressure, but also from studies in which organisms were shielded from the normal external pressure fluctuations for as long as three consecutive months.

Recent work (Figge, 1947; Brown, Bennett and Ralph, 1955) has suggested that some form of cosmic radiation might be capable of influencing organisms. This view has been strengthened by the discovery of 27-day organismic fluctuations (Brown, Bennett, Webb and Ralph, 1956), a frequency recently reported to exist also in fluctuations in cosmic radiation (Simpson, 1954). As a consequence, the following studies were undertaken to investigate in some detail any possible relationships between general background radiation and organismic metabolism.

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[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, NONR–122803.

[2] The authors wish to acknowledge their indebtedness to Professor H. T. Davis of the Department of Mathematics, Northwestern University, who gave freely very valuable advice during the course of the investigation and preparation of the material for publication.

MATERIALS AND METHODS

The O_2-consumption of potatoes was recorded continuously from February 1 through May 31 at Evanston, Illinois by means of Brown (1954) recording respirometers modified in such a manner (Brown, 1957), that a constant pressure was maintained by hermetically sealing the respirometers and recording system in rigid copper containers, the barostats, in which the pressure was kept at a constant reduced level of 28.50 inches Hg. Five barostats, each with four respirometers jointly providing a single continuous record of the fluctuations in rate of O_2-consumption, were in essentially continuous operation during the four-month period.

A cylindrical core of potato with an eye was placed in each respirometer. These cores were about 2.2 cm. in diameter and 1½ cm. high. The first lot was prepared on January 31, and with the exception of a very few occasional single-potato replacements continued in the respirometers until May 1 when a completely new set of potatoes was substituted. These latter were followed through the month of May. Therefore, the first lot of potatoes remained in constant conditions including pressure for three months except for brief periods of 15–20 minutes once every two to six days when the O_2 reservoirs were being refilled and the CO_2-absorbent renewed. The second lot remained in constant conditions, with no replacements for one month.

Only complete, uninterrupted calendar-days of recordings were used in the analysis. Partial days of data (days a respirometer was set up) were discarded.

Background radiation was recorded continuously during the same four-month period by means of a 2 × 30-inch cosmic-ray counter with an appropriate scaler and data printer. This monitoring system, located in the same laboratory with the five barostat-respirometer ensembles, yielded a count rate of the order of 40,000/hour. A few days of data were missed about the middle of May.

RESULTS

There were clear systematic fluctuations in O_2-consumption in the potatoes throughout the four-month period. These were most commonly ones appearing to possess a single major cycle a day. When 3 × 7-hour moving means of the average of all those two to five barostats for which recordings were complete on that day were calculated it was found that these daily fluctuations involved up to 28% increase, with a mean of 13.7% from lowest to highest values for the day for 30 sample days taken at random. In view of the leveling influence of the 3 × 7-hour sliding average, the actual range was undoubtedly substantially greater.

In Figure 1 (Nos. 1–5) are seen the mean forms of the daily fluctuation for the four-month period for each of the five barostats. It is quite evident that two general forms of mean daily fluctuation are apparent. Numbers 1, 3 and 4 showed a clear major cycle with a minimum in the early morning hours and a maximum in the late afternoon. Numbers 2 and 5 exhibited essentially a 180°-phase shift relative to the others. These five, independent, four-month samples, from lowest to highest values in the mean daily cycles are, respectively, 10%, 7.3%, 9.0%, 10%, and 4.8%. The average value of the five, 8.2%, is in remarkable agreement with the value, 8.0%, obtained for a two-month period in the spring of 1955 (Brown, 1957). All five mean cycles possess a minor peak about 6 P.M. and slight minima at 1–2 A.M. and 3 P.M., irrespective of the form of the major cycle. This fact is em-

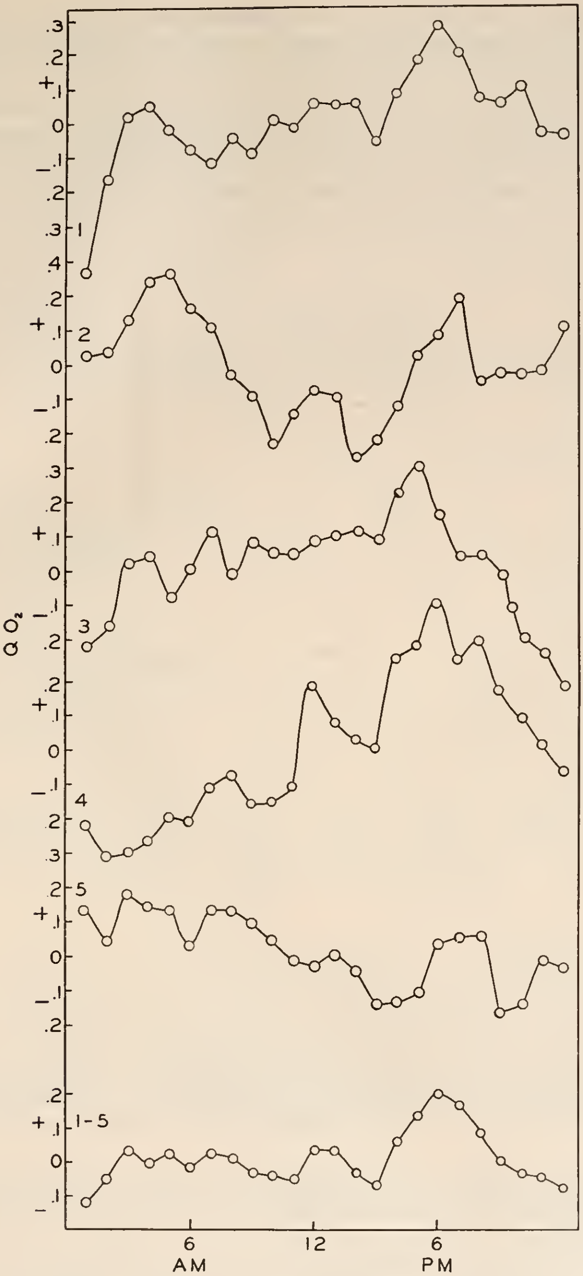

FIGURE 1. 1, 2, 3, 4, and 5 depict the mean daily cycles of fluctuation in O_2-consumption in potatoes, expressed as deviations from daily means, for each of the five independent respirometer-recording systems over the four-month period of study. 1–5 is the mean cycle for all the data. The actual percentage of the fluctuations is given in the text.

phasized in the mean 4-month cycle for all the barostats, Figure 1, (1–5) in which the 6 P.M. deviation from the daily mean is positive and highly significantly different from 0.

The mean daily cycle for all the barostats displayed no really significant evidence of a daily cycle except for the 5–6–7 P.M. peak clearly as a consequence of the algebraic summation of two forms of cycles, one essentially 180° out of phase with the other. It was apparent, also, from inspection of the single monthly mean cycles for the single barostats that each of the five mean four-month cycles included monthly cycles tending to be of the form of the final four-month mean form, or 180° out of phase with it.

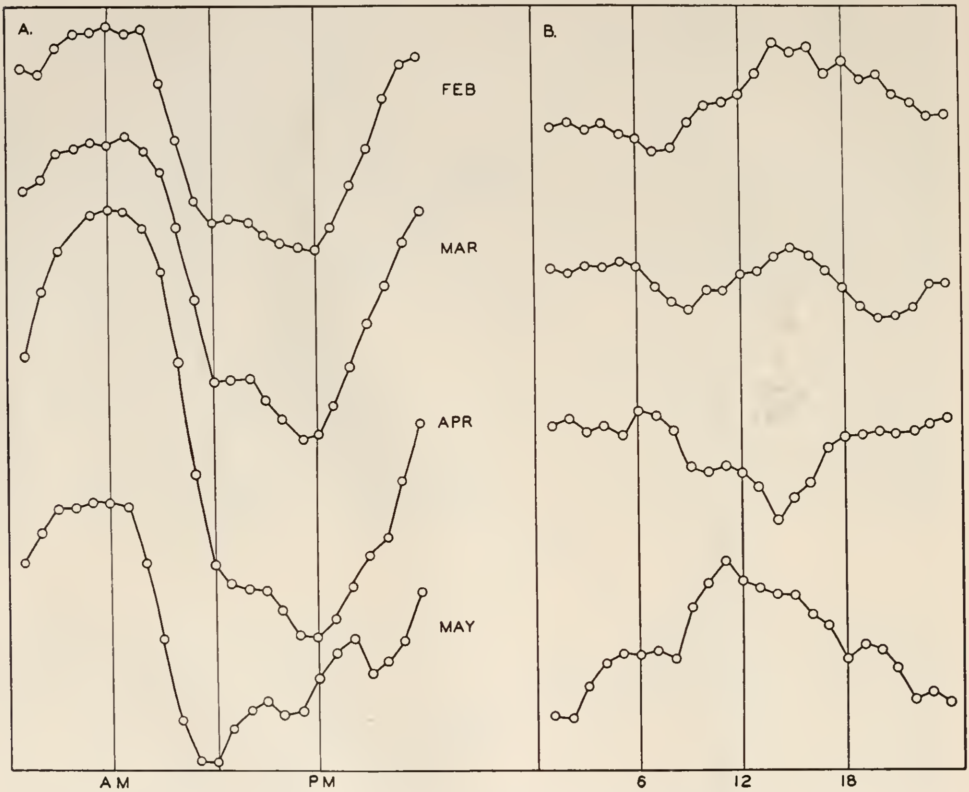

FIGURE 2. A. The mean daily cycles of general background radiation for each of four months. B. The mean lunar-day cycles of general background radiation for each of the four months. Lunar Zenith occurs at the 12th hour; Nadir, about 0 or 24. All these cycles are three-hour moving means of radiation. The mean percentages of the fluctuations are given in the text.

It seemed quite evident, therefore, that daily cycles of the potatoes were tending to exhibit one or the other of two forms, with, to the present, no suggestion that the occurrence of one type or the other is other than random.

An inspection of the fluctuations in radiation indicated there to be, in general, a clear mean daily cycle with a maximum about 6 A.M. and a minimum about 6

P.M. The mean daily cycles of three-hour moving means for each of the four months are seen in Figure 2 A. The mean cycles for February, March, and April are of strikingly similar form, with a gradually increasing amplitude (1.2%, 1.5%, and 2.3% increase from lowest to highest values). The maximum amplitude for a single day was about 10%. The cycle for May (5 days of data missing) showed an altered form though the amplitude, 1.3%, was of the same magnitude. Inspection of the daily data showed five days of the month (May 5, 15, 20, 23, 29) to have their cycles shifted about 180° relative to all the other days. The mean amplitude for the "typical" days was 2.2%, that for the five "shifted" days, 3.2%. Three days of the preceding month (April 1, 2, and 15) were also "shifted" days; no "shifted" days were present in February and March.

In view of the described presence of mean lunar-day cycles in numerous animals and plants, the mean lunar-day cycles of radiation were determined for each of the four months. These are seen in Figure 2 B. The cycles for February and March were rather similar, that of April shifted essentially 180° relative to the preceding two months. The cycle for May, with the highest amplitude of the four, again resembled those of February and March. There appeared to be a tendency for a maximum, or a minimum, to occur between the time of lunar Zenith and two to three hours afterwards, and for a maximum or a minimum to occur at the time of lunar Nadir. The amplitudes of the four average monthly cycles are, respectively, from minimum to maximum values, 0.57%, 0.37%, 0.60%, and 0.83%.

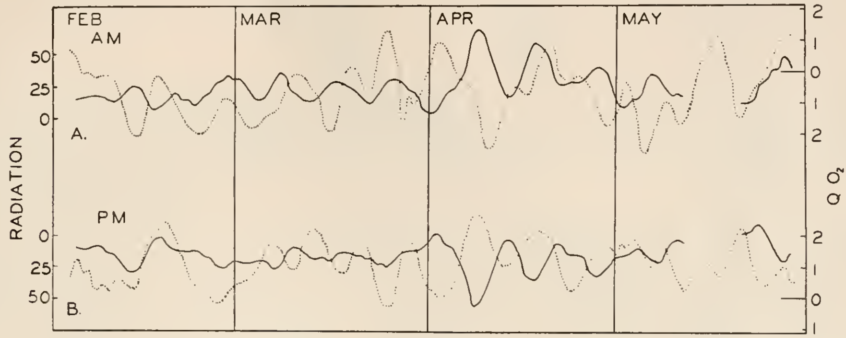

FIGURE 3. Five-day weighted (1:2:3:2:1) means of deviations of radiation intensity during the 2–6 A.M. and 2–6 P.M. periods from the daily means (solid lines) and corresponding weighted five-day means of O_2-consumption in potatoes for the 4–7 A.M. and 4–7 P.M. periods (dotted lines).

CORRELATION BETWEEN RADIATION AND O_2–CONSUMPTION

It is quite evident that even if the potato possesses mean daily cycles of O_2-consumption, its apparent tendency towards 180° phase-shifting would obscure much of this when large quantities of data were averaged. In view, however, of the relatively large-amplitude, daily fluctuations of the mean rates for all those potatoes recorded on a single day, an attempt was made to learn whether there might be a correlation between the amplitude of the day-by-day fluctuation in the background radiation and the amplitude of the day-by-day fluctuation in O_2-consumption of the potatoes.

In Figure 3 A is shown a weighted (1:2:3:2:1) five-day moving mean of the deviations in intensity of radiation from its daily mean for the 2–6 A.M. period (all were positive values), and similar deviations from the daily means for the 2–6 P.M. period (all were negative values). Plotted on different ordinate scales are superimposed correspondingly weighted five-day moving means of the deviation of O_2-consumption from the daily means for the 4–7 A.M. and 4–7 P.M. periods. It is evident from inspection that there is a highly suggestive similarity between the fluctuations in radiation and in O_2-consumption if one admits that the organismic cycles display alterations in sign of their correlation from time to time.

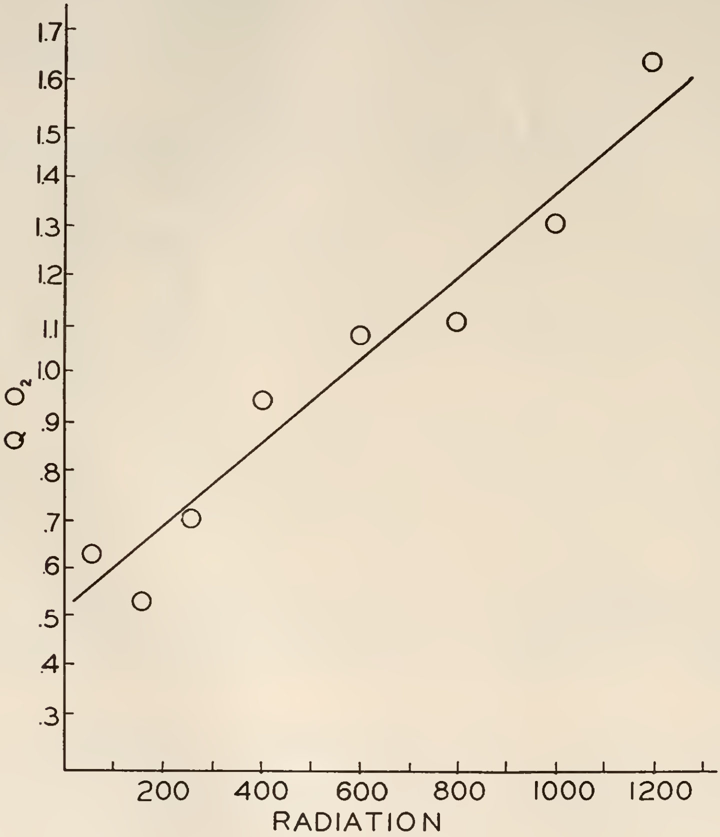

FIGURE 4. The relationship between the deviation in O_2-consumption at 4–7 A.M. and 4–7 P.M. from the daily mean of day *n*, expressed as deviations from monthly means, and the square of the deviation of radiation at 2–6 A.M. and 2–6 P.M. from its daily means for day *n*-1 expressed in the same terms ($P < 10^{-6}$).

To quantify this similarity and to obtain at least an approximate measure of the significance of such an apparent similarity, a coefficient was determined for the correlation between the deviations of radiation from its mean monthly deviations for each of the two times of day, on the one hand, and the deviations of O_2-consumption from their mean monthly deviations, for these times of day on the other. In this correlation, the signs of the deviations were ignored. A study of the regressional relationship indicated the relationship to be non-linear, and that the deviation in O_2-consumption was, instead, linearly related to the square of the deviation in radiation, and that the deviation in O_2-consumption on day *n* showed its correlations with day *n*-2, and especially *n*-1, of radiation with a rapid drop in coefficient to days *n*-3 and *n*. The coefficients and their errors for days *n* through *n*-3 were, respectively, 0.191 ± 0.066, 0.337 ± 0.062, 0.290 ± 0.064, and 0.135 ± 0.068.

The calculated regression for O_2-consumption on day *n* on the square of radiation for day *n*-1 is seen in Figure 4. The relationships between deviations in radiation and in O_2-consumption were calculated to be as follows:

Radiation	QO_2
0.5%	0.3%
1.0%	1.8%
1.5%	4.1%
2.0%	7.0%

The lag-lead relationship, radiation day *n*-1, was apparently simply the best compromise between radiation on day *n*-2 when using only the 4–7 A.M. value of O_2-consumption (0.41 ± 0.081), and radiation on day *n* using only the 4–7 P.M. value (0.369 ± 0.084).

An inspection of the form of the fluctuations in radiation and in O_2-consumption clearly suggested that in no other lag-lead relationship would correlations significantly different from zero be found over the four-month period. However, this was investigated more specifically. Correlations were found between radiation and O_2-consumption as follows: between the 4–7 A.M. deviation in O_2-consumption on day *n* from its mean 13-day deviation for this time of day, and the deviation of the 4–7 P.M. (day *n*-2) to 4–7 A.M. (day *n*-1) change in radiation from its mean monthly change for this period the coefficient was 0.352 ± 0.087. On the other hand, between the corresponding 4–7 P.M. deviation in O_2-consumption for day *n* and the corresponding 4–7 A.M. to 4–7 P.M. change in radiation of day *n* the coefficient was 0.382 ± 0.083. Eleven other lag-lead relationships for each of the times of day, sampling from day *n*-30 to day *n* + 15 for radiation failed to yield any correlations similarly highly significantly different from zero.

DISCUSSION

The results which have just been described provide one additional kind of evidence in support of the conclusion reached by Brown, Freeland and Ralph (1955) and Brown, Webb, Bennett and Sandeen (1955), in a study of correlations between metabolism of various organisms and concurrent rates and directions of barometric pressure change, that the organism in "constant conditions" still is responding to fluctuations in some external physical factor or factors. This view was given further support by the studies of Brown, Bennett, Webb and Ralph (1956) on the quahog. In more recent studies (Brown, 1957) employing barostats as in the current study, correlations with barometric pressure were found for O_2-consumption in potatoes over about a 1½-month period of study. In this latter investigation, unlike in earlier ones, there was a strong indication that the fluctuations in O_2-consumption on any given day tended to display either one of two patterns, one tending to be 180° out of phase with the other for either the whole or part of the day.

In the last-mentioned study there was a correlation of the deviations (ignoring sign) from the daily mean of the O_2-consumption of the potato at 4–7 A.M. on day *n* with the rate and direction of barometric pressure change from 2 to 6 A.M. centered on day *n*-2. In another kind of analysis of the data, there was found to be for the potato, a correlation between the 4–7 P.M. deviation from the daily mean of O_2-consumption and the mean barometric pressure on day $n + 2$ (Brown, Webb and Macey, 1957). Despite these correlations, the directly effective factor, in view of the use of barostats, could not have been pressure *per se*.

It is interesting that in this current four-month study there was also clearly reproduced a positive correlation between the deviation of the 4–7 P.M. value of O_2-consumption on day *n* from its daily mean and the mean barometric pressure of day $n + 2$ (Brown, Webb and Macey, 1957). It will be recalled that the 4–7 P.M. period was the only period of the day possessing a non-inverting cyclic component, and the correlation with barometric pressure correspondingly remained positive throughout the whole four-month period. In the spring of 1954, when a comparable and relatively striking negative correlation (– 0.65) was found using the potato (not in barostats), it was similarly only the 4–7 P.M. period of the day which possessed this property.

Another notable observation made in the current studies is that the correlation with the deviations in radiation were maximum for days *n* to *n*-2(*n*-2, for the 4–7 A.M. O_2-consumption) for radiation. This is essentially the same lead-lag relationship found for the potato in the spring of 1955, a year earlier, with rates of barometric pressure change for comparable periods of the day, and also which obtained during the summer of 1955 for the sea weed, Fucus (unpublished results).

It seems reasonable to postulate that the living organism is displaying a mean one- to two-day lag response to some external factor correlated both with fluctuations in barometric pressure and with daily cycles in background radiation, and that the effective external fluctuations are in some manner correlated with the mean daily barometric pressure on the third to fourth day thereafter. From the standpoint of a possible significance of these external factors for the maintenance of the precision of the many known regular daily cycles observed to persist for long periods under constant conditions, it must be admitted that the organism can exhibit a metabolic response to some external factor which has clear mean daily cycles, even though with a randomly fluctuating amplitude. It has been postulated earlier (Brown, 1957) that organisms, through an endogenous capacity to oscillate, are able to maintain in many instances an endogenous cycle of the same frequency as the external ones.

The mean lunar-day cycles of radiation are of special interest relative to lunar-day mean cycles of biological activities which have been described (*e.g.*, Brown, Freeland and Ralph, 1955; Brown, Webb, Bennett and Sandeen, 1955; Brown, Shriner and Ralph, 1956). The principal maximum (or minimum) in both the mean lunar-day cycles of radiation and of most of those biological activities so far described appear to occur at the times of lunar Zenith or shortly afterwards. The results encourage a more detailed study of lunar-day relationships of radiation and activity comparable to the current one for solar-day relationships. Such a study is in progress. Also, very suggestive in this regard is the observation that the ratio of amplitude of the mean solar-day cycles to the lunar-day ones of radiation, is of the same order as the ratio of the amplitude of the solar-to-lunar-day cycles of most of the reported mean cycles for organisms, namely 2 or 3 to 1.

SUMMARY

1. O_2-consumption of potatoes was recorded continuously through the four-month period, February-May, 1956.

2. There were daily fluctuations in rate which, even using 3 × 7-hour moving means, displayed a mean amplitude of about 14%.

3. Five independent respirometer-recording systems yielded mean daily cycles for the four-month period ranging in amplitude from 4.8 to 10.0% with a mean of 8.2%.

4. The mean cycles were of two forms, one essentially 180° out of phase with the other.

5. Solar-day and lunar-day mean fluctuations in background radiation were also determined for the period of the investigation.

6. A small but very highly significant correlation existed between the fluctuations in amplitude of the daily cycles in radiation on day *n*-1 and amplitude of the daily fluctuations in O_2-consumption in the potato on day *n*.

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LAG–LEAD CORRELATIONS OF BAROMETRIC PRESSURE AND BIOLOGICAL ACTIVITY[1, 2]

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It has been known for many years that numerous kinds of organisms representing most of the major divisions of living things exhibit under constant conditions overt cyclic fluctuations of solar-day frequency of one or more of their processes. Less well known is the fact that at least a few organisms which live in the intertidal regions of the oceans have similarly clear cycles of tidal frequency. These rhythms appear to be expressions of a biological-clock system upon which the organism normally relies importantly in the adaptive regulation of its physiological behavior in its rhythmic external environment. In the past few years it has been firmly established that the frequencies of these cycles are independent of temperature over a wide temperature range, a characteristic which is, of course, an essential one if these cycles were to have practical adaptive value in the normal environment with its substantial fluctuations in temperature from hour to hour and day to day. Particularly within the past two years it has become more and more evident that all animals and plants have average solar-day and lunar-day fluctuations and that in the maintenance of these cycles, the organisms are receiving stimuli of some character from the fluctuating external physical environment even under conditions generally considered to be constant.

Evidence for an influence of a fluctuating external factor affecting the organisms even under "constant conditions" has come from both 1) highly significant correlations between hourly rates of metabolism in several organisms and concurrent hourly barometric pressure changes, and 2) remarkable similarities in the forms of day-to-day changes in mean rates of metabolism for the whole day, or specific parts of a day, and the forms of the day-to-day large climatic changes in the mean daily barometric pressures (approximated by the pressure for any arbitrarily selected, restricted, time of day). The day-to-day drifting of mean barometric pressures in a temperate-zone area appears superficially to be random, with every given period a month or so long, exhibiting its own specific pattern. Despite this, the forms of the day-by-day fluctuations in rates of metabolism or certain other biological phenomena of a number of species of organisms have appeared to be rather similar, either in a direct relationship or an inverse one, to the concurrent fluctuations in mean daily pressures. This has been found true to such an extent

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as to seem more than fortuitous, though there is no generally acceptable test for significance of correlations of such time series.

For many years relationship between barometric pressure and human physiology has been believed to exist. As a result of the prevalence of such a view during the first half of the last century Vivenot (1860) performed what he considered a crucial experiment to test this view. He constructed an air-tight chamber within which an experimenter could study pulse and respiration rates in subjects while the pressure within the chamber was experimentally altered. With this he found no evidence that small pressure changes had any influence upon the individual. A few years later, Lombard (1887) described striking similarities between fluctuations in the strength of the normal knee-jerk and concurrent fluctuations in external barometric pressure and temperature. The correlation was positive with pressure and negative with temperature. Later, the same investigator (Lombard, 1892) in more extensive studies reported a correlation between the rate of fatigue for voluntary contraction of the flexor muscle of the second finger and barometric pressure. With rising pressure there was increased capacity for work and with falling, decreased. The biological phenomenon retained its correlation with both the regular daily tidal and the irregular climatic pressure changes. Furthermore, pressure changes experimentally obtained by ascending and descending a mountain yielded quite comparable correlations.

Some experiments with spontaneous activity of other mammals yielded comparable apparent relationships to barometric pressure changes. Hodge (1897), studying the spontaneous running of two dogs through the application of special pedometers to their collars, found a correlation between the mean daily activities of the two dogs with respect to one another and to the concurrent mean daily barometric pressure. With the latter the correlation was positive over the two-month period of study. The correlation with pressure was extensively confirmed by Stewart (1898) employing rats and certain other mammals, in activity recorders. Gray rats exhibited a negative correlation with pressure over the 70-day period of his study. White rats, for a 30-day period included within the previous study period, showed a positive correlation with pressure. Stewart postulated, on the evidence at hand, that wild mammals displayed a negative, and domesticated animals a positive, correlation with pressure.

A widespread occurrence of a correlation between barometric pressure changes and biological activities was found by Brown, Freeland and Ralph (1955) and Brown, Webb, Bennett and Sandeen (1955) who were investigating fluctuations in O_2-consumption in potatoes, carrots, seaweed, fiddler crabs, and salamanders. All of these organisms showed correlations highly significantly different from zero between the hourly rates of O_2-consumption and the concurrent rate and direction of barometric pressure change. Later, Brown, Bennett, Webb and Ralph (1956) found correlations between the spontaneous opening of oysters and quahogs, and barometric pressure changes. In these recent studies it was strongly suggested by inspection of the data, however, that in paralleling the patterns of fluctuations, the organisms were actually leading the barometric pressure changes by more than a day.

One purpose of this report is to describe results from a simple statistical analysis of all the results on this score obtained in our laboratories between March 1, 1954 and June 9, 1955. These point conclusively to an ability of a wide variety of living

TABLE I

Organism and place	Dates (inclusive)	Correlation times Organism/Bar. pressure	r
Fucus[1] Woods, Hole, Mass.	July 28–Aug. 2 } 1954 Aug. 21–29 }	day n (5–7 A.M.) / $n + 2$ (5–7 A.M.)	-0.476 ± 0.200
	Aug. 3–20, 1954	day n (5–7 A.M.) / $n + 2$ (5–7 A.M.)	$+0.507 \pm 0.176$
Ostrea (10° C.)[2] Evanston, Ill.	Mar. 1–April 13, 1954	day n (av. daily) / $n + 1$ (av. daily)	$+0.411 \pm 0.125$
Ostrea[3]	June 18–July 28, 1954	day n (av. daily) / $n + 2$ (av. daily)	$+0.383 \pm 0.139$
	July 29–Aug. 27, 1954		-0.658 ± 0.104*
Rattus[5]	Nov 16–Dec. 3 } 1954 Feb. 2–Mar. 13 } 1955	day n (noon) / $n + 7$ (noon)	$+0.598 \pm 0.068$*
	Dec. 5–Feb. 1, 1954–55	day n (noon) / $n + 7$ (noon)	-0.362 ± 0.113
Solanum[1] Evanston, Ill.	May 12–June 9, 1954	day n / $n + 1$	-0.650 ± 0.110*
Solanum[6] Evanston, Ill. (5 groups)	April 1–June 8, 1955	day n (5–7 P.M.) / $n + 2$ (5–7 P.M.)	$+0.266 \pm 0.071$
Triturus[4] Evanston, Ill.	May 12–June 9, 1954	day n (5–7 P.M.) / $n + 2$ (5–7 P.M.)	-0.842 ± 0.059*
Uca pugilator[4] Woods Hole, Mass.	June 20–July 20, 1954	day n (5–7 A.M.) / $n + 2$ (5–7 A.M.)	-0.472 ± 0.144
	July 21–Aug. 27, 1954	day n (5–7 A.M.) / $n + 2$ (5–7 A.M.)	$+0.595 \pm 0.104$*
Uca pugnax[4] Woods Hole, Mass.	June 18–Aug. 29, 1954	day n (5–7 P.M.) / $n + 2$ (5–7 P.M.)	-0.423 ± 0.100
Venus[3] Woods Hole, Mass.	June 18–Aug. 29, 1954	day n (av. daily) / $n + 2$ (5–7 A.M.)	-0.446 ± 0.096

[1] Brown, Freeland and Ralph (1955).

[2] Brown (1954).

[3] Brown, Bennett, Webb and Ralph (1956).

[4] Brown, Webb, Bennett and Sandeen (1955).

[5] Brown, Shriner and Ralph (1956).

[6] Brown (1957).

* Conventionally determined standard errors for such large values of r do not provide true measures of probabilities.

things, ranging from lower to higher plants and from lower to higher animals, to show a lead correlation with barometric pressure changes by one to seven days (usually two). The only organism studied in our laboratory during this period which was not included in this report is the carrot, which was omitted, not because of any lack of similar type of correlation, but rather because there were some known injury effects on the O_2-consumption during part of the single-month period of study.

The organisms, the times of study, and the coefficients of correlation obtained in 703 organism-days are given in Table I. These are all correlations of three-day moving means except for the potato in 1955, in which daily mean values of O_2-consumption were correlated with three-day sliding averages of barometric pressure. Indicated in footnotes are the references to the publication of the results obtained with these species. In these publications it had not occurred to the authors to make this type of analysis, and furthermore, due to the extraordinary nature of the conclusions, it is to be doubted that a single demonstration of the phenomenon of a lead correlation, even though shown to be statistically significant (assuming random fluctuation in the organism), would have been credited by most physiologists.

All of the results were obtained in conditions of constant, continuous low illumination of the order of 1 ft. c. or less, except with the white rat, *Rattus,* for which two periods of continuous darkness, totalling 43 days, were included in the data. The conditions of the experiments with the brown alga, *Fucus,* two species of fiddler crabs, *Uca pugnax* and *Uca pugilator,* the salamander, *Triturus* and the potato, *Solanum* included also very precisely regulated constant temperature, and, in addition, for *Solanum* in 1955, constant pressure through the use of a barostat.

The biological process studied was the rate of O_2-consumption in *Fucus, Uca pugnax* and *Uca pugilator, Triturus* and *Solanum.* It was the average daily minutes open per hour for both the oyster, *Ostrea* and the quahog, *Venus;* it was the total distance spontaneously run per day in the case of *Rattus.*

To determine when the correlation would be highest, whether with the same day (day n) of barometric pressure, the following day (day $n + 1$), the second day after (day $n + 2$), or some earlier or later one, tracings of the fluctuations of the physiological process and of barometric pressure were superimposed and inspected in various temporal relationships. In the vast majority of cases, there was clearly only one relationship which promised high correlation with an obviously rapid drop towards no correlation with either greater or less displacement and no other relationship with nearly as high a correlation could be seen by such inspection over the rest of the period. In two cases, *Triturus* and *Solanum,* it was not evident from inspection, whether the correlation would be best with day $n + 1$, $n + 2$, or day $n + 3$. In these cases, coefficients were determined for all three times and the highest one was selected; it was obvious, however, there was no other relationship with these species in which the correlation would be as high or significant.

The change in sign of the correlation from time to time seems clearly to be a biological contribution, probably to be compared superficially with the well-known changes of sign frequently observed in animal orientation. The sign change appears to occur abruptly and cleanly; the cause of the change is still unknown. It is known, however, that in all those cases examined in which the sign of correlation changed, there was concurrently a transformation of the form of the mean daily and lunar-day cycles to essentially their mirror images.

For all the periods the correlations ranged in size from 0.266 to 0.842. All were significantly different from zero by ordinary tests for correlations. Both the − 0.362 for *Rattus* and the 0.383 for *Ostrea* appeared to include one or two very brief periods of change of sign in the relationship. The potatoes in 1955, judging by periodic inversions of their solar-day cycles, appeared to be changing the sign of their correlation from time to time, and probably hence the lowest, though quite real, correlation.

The great majority of the correlations centered on day $n + 2$ of barometric pressure. *Solanum* in 1954 and *Ostrea* at 10° C., centered on day $n + 1$. The potato in 1955 gave almost the same correlation for $n + 2$ and $n + 3$. The most surprising result, on the basis of hypotheses available to account for this phenomenon, was that *Rattus* showed by far its highest correlation with day $n + 7$. The coefficient would have risen from 0.598 ± 0.088 to 0.668 ± 0.07 by the justifiable statistical procedure of eliminating from consideration the transitional values, those of Dec. 1–3 and Feb. 2, which were seen clearly to contribute naturally to neither series.

Since there appeared to be no test for the significance of correlations between two time series that would be acceptable to all statisticians, further experiments were performed to attempt to demonstrate the reproducibility of the phenomenon. The first of these involved a four-month study of potatoes in Evanston, Illinois. Small cores of potatoes bearing eyes were obtained by means of a large cork-borer, and one was placed in each of 20 respirometer vessels (Brown, 1954). Four respirometer vessels upon a single recording system were sealed in each of five barostats on Feb. 1, 1956. During a three-month period, the respirometers were opened for about 15 minutes once every two to six days, to refill the O_2-reservoirs and replace the CO_2-absorbent. Very rarely a potato was replaced with a new one during this period. On the first of May, a completely new lot of potatoes replaced the old, and the observations were continued through May 31. During this study the potatoes were maintained in constant conditions of temperature (19.5° C.), of light (< 0.5 ft. c.), and of all other factors known to influence organisms. The respirometers were kept under a constant reduced pressure of 28.50 inches Hg. Approximately 56,000 organism-hours of O_2-consumption were obtained.

Inspection of three-day moving means of the mean daily barometric pressure for the four-month period, and comparison of weighted (1:2:3:2:1) five-day moving means of the 4–7 P.M. deviation in rate of O_2-consumption from the daily mean, gave clear suggestion that just as with the potatoes in the 1954 and the 1955 experiments, there was a lag correlation of barometric pressure on O_2-consumption by about two days. A scatterplot of the relationship between the barometric pressure of day $n + 2$ and O_2-consumption on day n is seen in Figure 1. This yielded a correlation coefficient of 0.339 ± 0.0835, a value highly significantly different from zero.

Figure 1, B, illustrates the various values of r obtained in various lag-lead relationships between the two phenomena, *i.e.,* for O_2-consumption of day n correlated with barometric pressure in various temporal relations from day $n - 30$ to day $n + 15$, a 45-day span. In this instance not only was a correlation centered

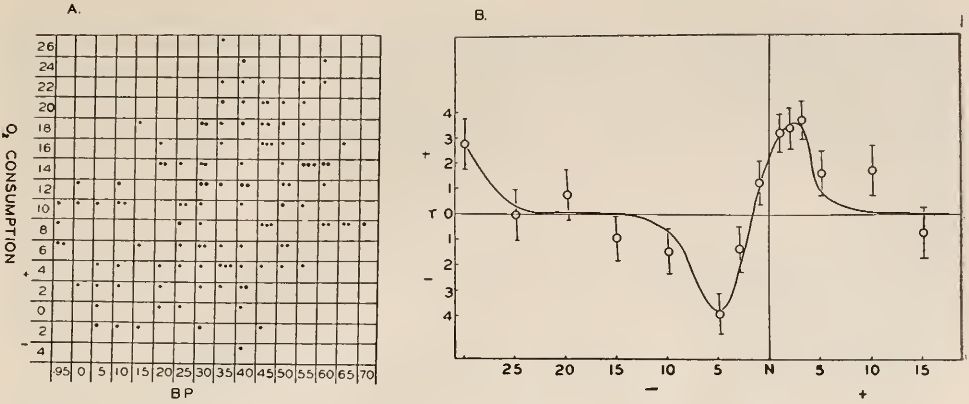

FIGURE 1. A. Scatterplot of the relation between the mean barometric pressure on day $n+2$ and the 5–6–7 P.M. deviation from the daily mean of O_2-consumption of the potato on day n. B. Coefficients of correlation (ordinate) between the 5–6–7 P.M. deviation in O_2-consumption from the daily mean on day n and the mean barometric pressure on various days from $n-30$ to $n+15$ (abscissa).

on day $n+2$, but a correlation was also found with barometric pressure, day $n-5$.

A second attempt was made in 1956 to confirm an organismic lead-correlation of metabolism on barometric pressure. This one was performed in Woods Hole, Mass., between June 16 and August 1. These observations were made upon fiddler crabs, whose O_2-consumption was measured under constant conditions including pressure in the same type of apparatus as that used for the potatoes. In this study of the fiddler crab, the temperature was similarly very constant but at a higher

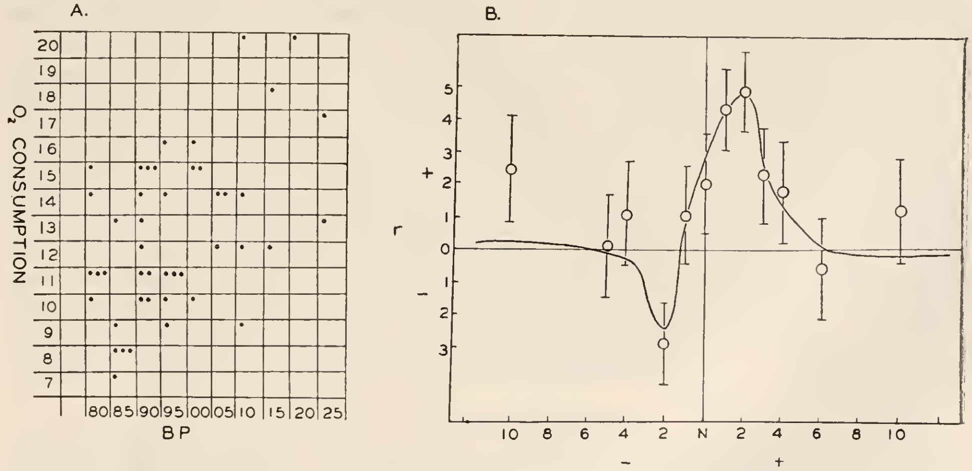

FIGURE 2. A. Scatterplot of the relation between the mean barometric pressure on day $n+2$ and the 5–6–7 P.M. value of O_2-consumption of the fiddler crab on day n. B. Coefficients of correlation (ordinate) between the 5–6–7 P.M. value of O_2-consumption from the daily mean on day n and the mean barometric pressure on various days from $n-10$ to $n+10$ (abscissa).

value, near 24° C. In this analysis, as for the experiments performed in 1954 and 1955, the actual rates of O_2-consumption at the 4–7 P.M. period were used. The scatterplot relationship between respiration on day n and barometric pressure on day $n + 2$ is shown in Figure 2, A. This yielded a coefficient of 0.49 ± 0.12, remarkably close, coincidentally, to the mean of all the values obtained in 1954 and 1955 (0.5). In Figure 2, B, are depicted the values for r together with their deviations for this and other lag-lead correlations between day n of O_2-consumption and day $n - 10$ to $n + 10$ for pressure. The only real correlations in the series are for days $n + 1$ and $n + 2$, with the latter being the more highly significant. In these correlations with the crabs three-day moving means were used for both pressure and metabolism.

These two series of experiments performed in 1956 would appear to confirm in a striking manner the conclusions reached in the earlier studies.[3]

The explanation of the phenomenon considered here seems from a general standpoint to be quite evident. Since the organisms cannot be determining the barometric pressure changes which are to occur, the organisms must be responding to some physical factors and their fluctuations which themselves exhibit a lead-correlation on barometric pressure. The potatoes in 1955 and the potatoes and crabs in 1956 remained in each case in barostats at 28.50 inches Hg from one to three months, the barostats opened only for about 15 minutes once every two to six days, and hence, the lead correlation cannot be due to any special responses to current rates of change in pressure itself. Since organisms have been shown to possess fluctuations in metabolic rates correlated with the rates and directions of barometric pressure change and especially since 27-day cycles have been found, it appears suggestive that the organism may be able to respond directly to fluctuations in the intensity of some high energy radiation or of some other physical factor with radiation-correlated fluctuation.

It is futile at the present time to do much speculating as to the external forces involved and the manners in which fluctuations in them may interact with the now established solar-day and lunar-day clocks and average cycles within organisms. This is now being investigated. It may be shown eventually that the phenomenon described in this report depends in some manner on the possession by both the organisms and the atmosphere of a solar-day and lunar-day cyclicity, and the interaction of these with some less orderly cosmic factor to which both the organism and atmosphere can react in an oscillatory fashion. But irrespective of the detailed mechanism, correlations of the order of magnitude described here (nearly 0.50 as the average degree of correlation for eight species of animals and plants over about 850 days), and tending very strongly to be centered on day $n + 2$, especially since they cannot be correlations with an actual causative force, are to be viewed as extraordinary. They force one to conclude that the living organism is clearly responsive in an orderly way to forces not hitherto seriously considered by biologists to possess any influence.

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[3] Since the manuscript was completed, a further study of the potato during October, November and December, 1956 also yielded a lead correlation in which the highest correlation (-0.400 ± 0.089) was similarly found with the mean barometric pressure of day $n + 2$, rapidly falling on days $n + 1$ and $n + 3$ to -0.307 ± 0.094 and -0.328 ± 0.093, respectively, and on days n and $n + 4$ to a value not significantly different from zero. From inspection there was no other lag or lead relationship in which a significant correlation existed.

For the biologist who is attempting to account for the remarkable capacity of organisms to measure off with great precision under so-called "constant conditions," temperature-independent cycles of the frequencies of natural external cosmic events, it becomes highly important to know whether the conditions are truly constant for the organism, and if not, what is the actual character of the fluctuations in the effective external factor or factors.

SUMMARY

1. Eight species of living things, ranging from lower to higher plants and lower to higher animals, in studies over a three-year period and including approximately 850 species-days have exhibited without exception a statistically significant lead-correlation on barometric pressure with an over-all mean coefficient of about 0.5.

2. The correlation involved sometimes only the 5–6–7 A.M., sometimes only the 5–6–7 P.M., and other times the mean daily rates of O_2-consumption.

3. The sign of the correlation was sometimes positive and other times negative. Sign changes, when they occurred during a single period of study were abrupt, and correlated with a 180°-shift in the phase relationships of the concurrent mean solar-day cycles.

4. In twelve periods of study, ranging from one to four months each, the correlation in nine cases centered on day $n + 2$ of barometric pressure. In two cases it centered on day $n + 1$ and in one, on day $n + 7$.

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THE LUMINESCENCE OF THE MILLIPEDE, LUMINODESMUS SEQUOIAE [1]

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The millipede *Luminodesmus sequoiae* was first described by Loomis and Davenport (1951). Following this, Davenport, Wootton and Cushing (1952) described the biology of the animal and the general nature of its luminescence. They found that light emission originates from cells in the deeper layers of the integument. The present paper describes a more detailed study of its luminescence.

The bioluminescent reaction in four different organisms (*Cypridina,* fireflies, bacteria and *Gonyaulax*) has been studied in recent years (Tsuji, Chase and Harvey, 1955; McElroy and Hastings, 1955; McElroy and Green, 1956; Hastings and McElroy, 1955; Strehler, 1955; Hastings and Sweeney, 1957). The common feature is that the reaction involves an enzymatic oxidation with molecular oxygen. Although it was demonstrated by McElroy that adenosine triphosphate is an absolute requirement for firefly luminescence, its possible role as an energy source in the reaction has not been clarified. In bioluminescent reactions in general it is assumed that the energy must be derived from the oxidation of a substrate, which is usually termed luciferin (*e.g., Cypridina* luciferin, firefly luciferin, etc.). None of the products of luminescent reactions have been definitely identified and the reactants as well as the enzymes are different in all cases studied. Such studies are of interest for the general problem of how the living cell transforms chemical energy into other forms of energy.

Materials and Methods

About 1000 animals were collected by a party of five on the nights of May 10 and 11, 1956, in the vicinity of Camp Nelson, Tulare County, California. The animals were abundant and readily visible to the dark-adapted eye by their own light on the surface of the ground in the forest. The animals were brought back to the laboratory and stored in glass containers with ample humus. Light intensity was measured with apparatus previously described (Hastings, McElroy and Coulombre, 1953), using a photomultiplier tube and automatic graphic recording.

Results

1. In vivo luminescence

Luminescence in *Luminodesmus* is continuous but fluctuating. The light intensity (recorded from single animals over long periods up to 24 hours) fluctuates

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[1] This study was supported by grants from the National Science Foundation, the Graduate School of Northwestern University, and the Research Committee of the University of California, Santa Barbara College.

by 20 to 40 per cent (or occasionally more) around a relatively constant mean. Since these light intensity changes could be detected with the eye, we are certain that they do not result from movements of the animal. A two-hour portion from such a recording is reproduced in Figure 1A. It can be seen that there is an instance when the light intensity doubled, apparently spontaneously. Such a marked increase in light intensity also occurs when the animal is handled. Indeed we found that striking the test tube in which the animal was placed would evoke such a response (Fig. 1B). The response still occurred immediately after the animal had been decapitated. Upon stimulation of the nerve cord which induced electrical shocks no luminescent response was observed which could be attributed to the effect of the electrical stimuli. The way in which the luminescent changes are brought about in the living animal is not clear.

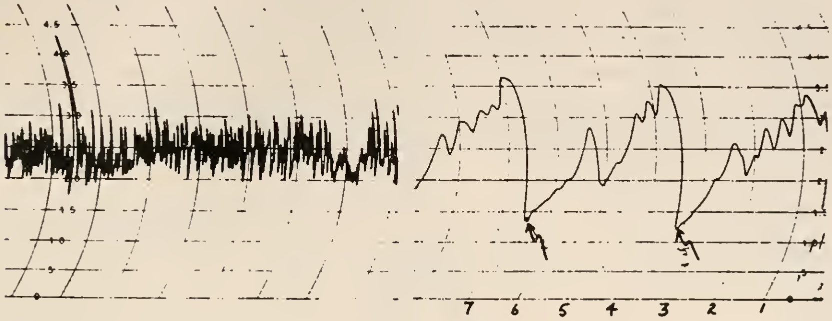

FIGURE 1. Reproductions of recordings of *Luminodesmus* luminescence. Ordinate, light intensity; abscissa, time, to be read from right to left in both cases. *Left:* Luminescence of an undisturbed animal over a two-hour interval. Time between vertical divisions, 15 minutes. *Right:* Luminescence changes of an animal where the test tube containing the animal was tapped lightly at the two instances noted by arrows. Time between vertical divisions, one minute.

Isolated pieces of the animal retain their luminescence for a long time. The intensity decreases gradually to about one half of its original value in 8 hours. Although fluctuations in intensity may occur for the first 15 minutes, it is essentially a steady luminescence thereafter. An eviscerated specimen from which the first few and last few segments are cut off behaves in essentially the same manner. Animals removed from humus and kept in a test tube for a day or more also showed little fluctuation in intensity. Whether or not this was due to water depletion or to starvation was not determined. Preparations with little or no light intensity fluctuation were used in the various experiments described below.

2. *Possibility of luminous symbiotic bacteria*

In some organisms luminescence arises from an association with luminous bacteria (see Harvey, 1952). The possibility that this might be the case in *Luminodesmus* was investigated some years ago by W. D. McElroy and one of us (J. W. H.). Whole animals and extracts of animals were put on agar plates containing a variety of media. Plates with a range of salt concentrations (0, 1%, 2% and 3% sodium chloride) were made up with both glycerol and glucose as carbon sources and Bacto Tryptone. No growth of luminescent bacteria occurred on any plate.

It may also be noted that the effect of varying oxygen concentration upon the luminescence of *Luminodesmus* (see section 5) indicates that the light is not bacterial in nature. In bacteria (Hastings, 1952; Shapiro, 1934) decreasing the oxygen concentration has no effect upon luminescence unless the concentration is less than about 0.3%.

3. Color of the light

The light emission from *Luminodesmus* is weak, requiring dark-adaptation on the part of the observer to see it. The emission spectrum was determined by placing a single animal at the entrance slit of a Bausch and Lomb Grating Monochromator, and the phototube at the exit slit. A second phototube was placed by the entrance slit to monitor any changes in the intensity of the animal during the course of the experiment. The entrance and exit slits were both set at 1 mm., which gives a dispersion of 12 mμ with the grating used (15,000 lines per inch). The light intensity was measured at the various wave-length settings and then corrected for phototube sensitivity and for monochromator efficiency. The corrected values are plotted against wave-length in Figure 2, giving the emission spectrum with the maximum in the green at 495 mμ. This spectrum is similar to that of some of the

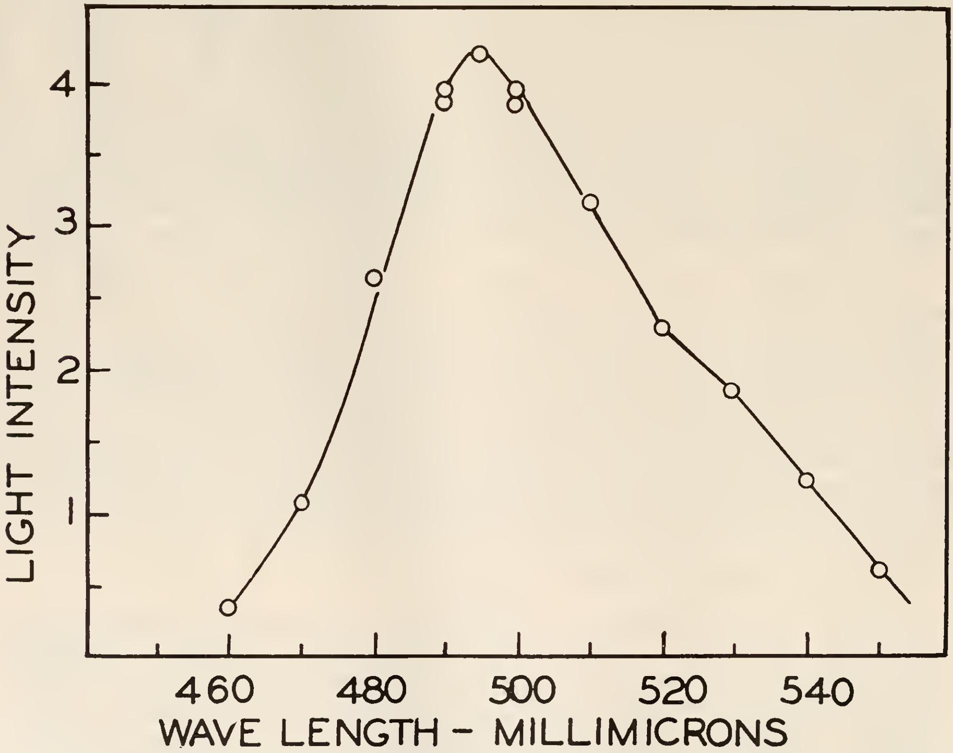

Figure 2. Emission spectrum of *Luminodesmus sequoiae*. Ordinate, light intensity in arbitrary units, corrected for phototube sensitivity and monochromator efficiency.

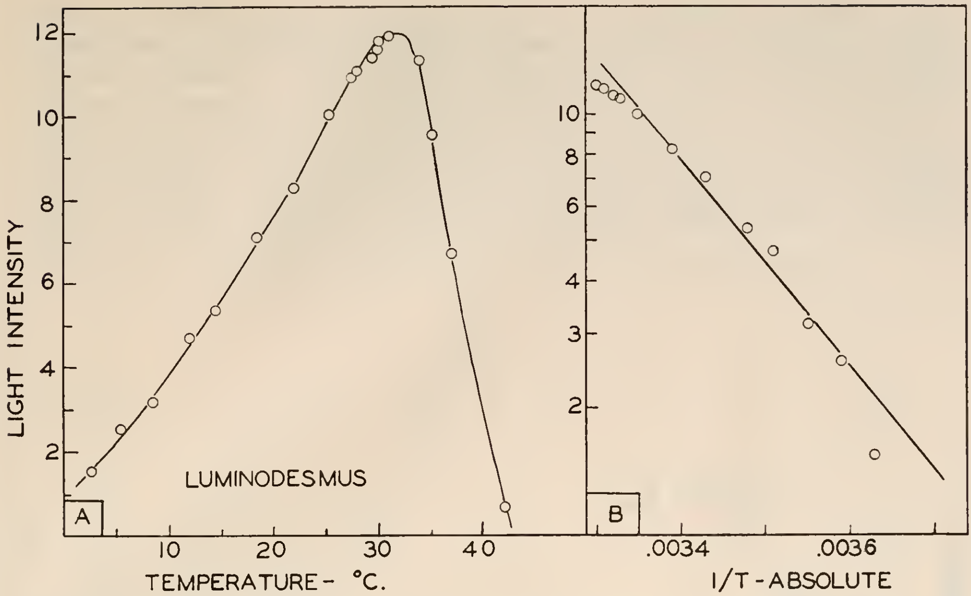

Figure 3. (A) Effect of temperature upon luminescence. Ordinate, light intensity in arbitrary units. See text for details. (B) Data of Figure 3A plotted according to the Arrhenius equation. Ordinate, light intensity in arbitrary units plotted on a log scale; abscissa, the reciprocal of absolute temperature.

luminous bacteria, although the spectrum of bacterial emission may depend upon the density of the suspension (Harvey, 1952).

4. *Effect of temperature*

The effect of temperature upon the luminescence was determined using both intact organisms and eviscerated specimens. The results were essentially the same in both cases. The specimen was held in place in a test tube by a cotton plug. Temperatures were adjusted by holding the tube in a water bath, and the temperatures plotted are those read from a thermometer placed in the tube beside the specimen. The tube was then quickly removed from the bath and placed in front of the phototube.

The data from one experiment with an eviscerated specimen are plotted in Figure 3, along with a plot of the data according to the Arrhenius equation. The Q_{10} for the process is 1.95 between 10° and 20°; 1.73 between 15° and 25°; and 1.55 between 20° and 30°. The sharp decrease in luminescence above the optimum of 31.5° is most likely the result of heat denaturation of enzymes. The activation energy for the over-all process may be calculated from the slope of the straight line drawn in the Arrhenius plot. The value obtained in this case is about 12,000 calories.

5. *Effect of varying oxygen concentration*

The effect of oxygen concentration upon the luminescence was determined quantitatively, using both whole animals and eviscerated specimens. Both gave similar results. Luminescence was greatest in 100% oxygen and progressively decreased at lower concentrations, being reversibly extinguished in pure nitrogen, as reported by Davenport, Wootton and Cushing (1952). An oxygen concentration of about 6.5% decreased the intensity from that in air by about one half.

The specimen was held in place with cotton in a stoppered 10-ml. test tube, with glass tubing to bring the gas mixtures into the test tube. Gas mixtures of the desired oxygen concentration were prepared by mixing nitrogen with air or oxygen at measured rates, using calibrated flow-meters. The typical effect of lowered oxygen concentration upon luminescence is shown in Figure 4. The response is essentially similar to that found in several other luminous organisms (Hastings, 1952; Hastings, McElroy and Coulombre, 1953; Hastings and Buck, 1956). There is a characteristic "undershooting" when the animal is exposed to a lowered oxygen concentration, and an overshoot or excess flash of luminescence, when it is exposed to a higher concentration. This excess luminescence is greater when a lower oxygen concentration is used during the period previous to the time when air is readmitted. For example, in the experiment shown in Figure 4, the luminescence was about twice the baseline level when air was admitted. With

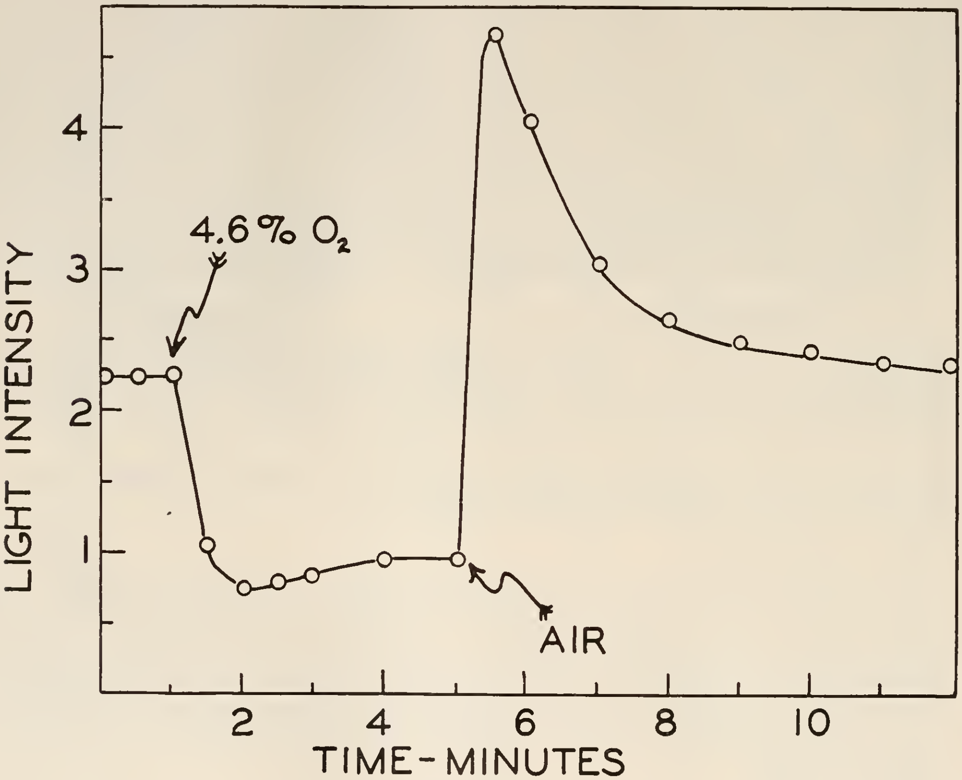

Figure 4. Changes in luminescence with time when a gas mixture containing 4.6% oxygen was passed over the animal for four minutes followed by the readmission of air.

1% oxygen the luminescence was 2.5 times and with 10% oxygen it was 1.5 times the baseline level. This suggests that the substrate for the luminescent reaction (luciferin) is the product of a series of relatively slow reactions. When the oxygen concentration is changed the luciferin comes to a new steady-state concentration, but only relatively slowly.

The values for light intensity versus oxygen concentration plotted in Figure 5 are the steady-state values, measured just previous to the time when the animal was returned to air. The data plotted are the results obtained with six different specimens. Although there was a variation in the results, the data for any given animal fall along a smooth curve, suggesting a real difference between one animal and another, rather than error in the method. The reason for these differences could not be ascertained. Neither carbon monoxide nor carbon dioxide when added to the gas mixtures in 1% concentrations had any effect upon the shape of the curve for a given animal.

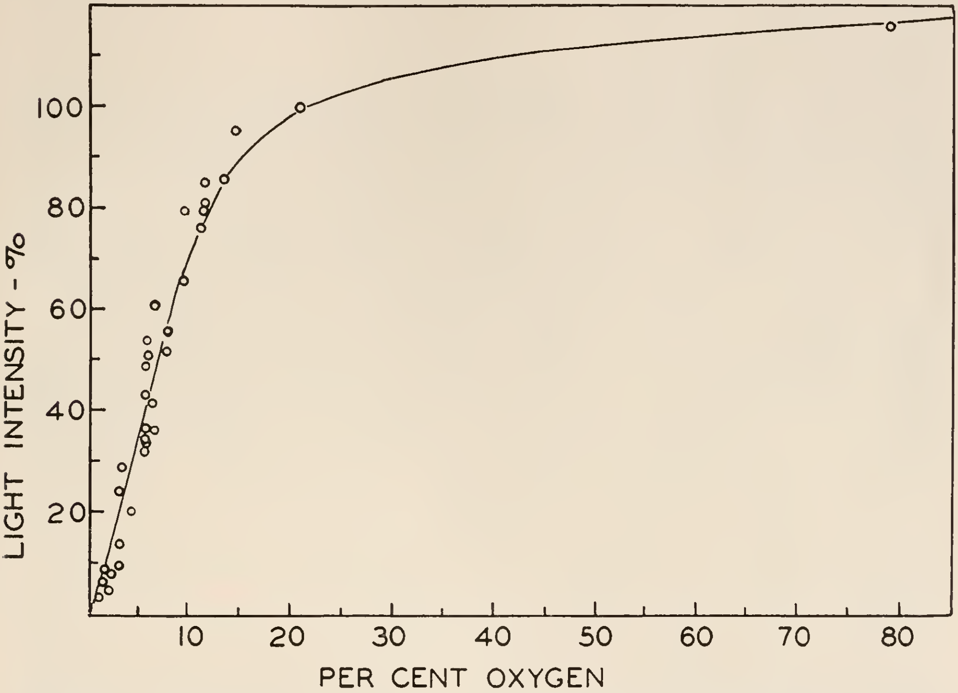

FIGURE 5. Effect of oxygen concentration upon the steady-state luminescence of *Luminodesmus*. Data are taken from experiments such as are illustrated in Figure 4. The luminescence, in low or high oxygen just prior to the readmission of air, is expressed as the per cent of the steady-state of luminescence in air.

6. Fluorescence

Luminodesmus is highly fluorescent under ultraviolet light, and it was suggested previously (Davenport *et al.*, 1952) that this fluorescent compound might be involved in the luminescent reaction. In luminous bacteria, for example, it has been demonstrated that reduced flavin mononucleotide ($FMNH_2$) is involved in the luminescent reaction, possibly as luciferin (McElroy, Hastings, Sonnenfeld and Coulombre, 1953; Strehler, Harvey, Chang and Cormier, 1954). FMN and other flavins are highly fluorescent in the oxidized form and are not at all fluorescent in the reduced form. Under anaerobic conditions in the intact animal the luciferin should be essentially 100% in the reduced state. However, there is absolutely no change in the fluorescence of *Luminodesmus* during anaerobiosis. The possibility that the fluorescence of the organism comes from a flavin compound involved in the luminescent reaction must therefore be ruled out.

7. Luminescence in extracts

Davenport, Wootton and Cushing (1952) reported negative results in all attempts to restore luminescence in filtered water extracts of *Luminodesmus*. Using sensitive light-measuring equipment, we have repeated their experiments and made additional studies with extracts. The most suitable method which we found for preparing extracts was to remove the gut from animals and dry them overnight in a vacuum desiccator with calcium chloride. The dry animals were then pulverized to a fine powder by grinding, and extracted with cold acetone. The acetone was removed by filtration and the dry powder kept in a vacuum desiccator. The powder remained active in this state for a period of at least two weeks.

When this powder was mixed with water a dim luminescence occurred (visible only to the dark-adapted eye), lasting for about 10 minutes, the half time for decay being about two minutes. Stirring always resulted in a temporary increase in luminescence, suggesting that leaching from particulate matter was taking place. Following stirring the light intensity returned to the original level. When such a solution was filtered through fine sintered glass (maximum pore size, 5.5 microns), the filtrate retained luminescence. Stirring did not affect the intensity of the filtrate, indicating that the particles from which leaching was occurring had been removed.

The intensity of luminescence in these extracts was dependent upon the pH of the solution, with an optimum at about pH 8.9. The determination was made by extracting equal quantities of the powder with 0.05 *M* trihydroxyaminomethane-maleic acid buffer at various pH values and measuring the light intensity of the solution. In all experiments described below a buffered extract at pH 8.9 was used, and buffered reagents where needed.

In the classical luciferin-luciferase test a fraction which has been extracted with hot water is combined with a cold water extract in which the luminescent reaction has been allowed to run to completion. In the hot water extract the enzyme has been destroyed, presumably leaving available substrate, or luciferin. In the cold water extract the luciferin has all been used up leaving active enzyme, or luciferase. The two mixed together should therefore give light, but with *Luminodesmus* completely negative results were obtained. Moreover, neither the hot water extracts nor exhausted cold water extracts, when added to a luminescing extract, had any effect upon the light intensity.

Attempts to separate the presumed luciferase and show its activity in the reaction were negative. Fractions were obtained by ammonium sulfate precipitation, by alcohol fractionation, and by dialysis. None was active when added either to luminescing mixtures or to hot water extracts.

A large number of compounds were tested for their ability to modify the light intensity in luminescing extracts.[2] ATP was found to have appreciable activity. ATP added to an extract which was emitting light caused the intensity to increase by 10 to 30 per cent. The effect was not due to a pH change since buffered ATP solutions were used. It was effective with filtered extracts as well as unfiltered. Other pyrophosphate compounds were not tried, so it is possible that the action of ATP could be non-specific, similar to the effect of pyrophosphate compounds added secondarily to luminescing firefly extracts (McElroy, Hastings, Coulombre and Sonnenfeld, 1953). The fact that ATP would not restore luminescence to dark extracts indicates such a non-specific role. No restoration of luminescence occurred even when ATP was added to hot water extracts together with exhausted cold water extracts. However, when $MgSO_4$ was added following ATP addition, there was an additional increase in light intensity, suggesting the possibility of a more specific role for ATP. Also, 0.05 *M* Versene (ethylene diamine tetra acetic acid) was found to depress luminescence, indicating the possibility that the reaction is activated by a metal ion. All of the coenzymes listed in footnote 2 were tested in combination with ATP. None was found to have any stimulatory effect, although FMN and riboflavin slightly depressed luminescence.

The results give little clue as to the nature of the reaction. If a luciferin type compound is involved in a classical oxidative reaction, possibly in combination with ATP, then the luciferin must be highly unstable. In fact, such a highly unstable and heat-labile luciferin could account for the results we have obtained. The inhibition by flavins might mean that some flavin compound is involved in the reaction, although we would not expect a flavin to be particularly unstable.

SUMMARY

1. The luminescence of *Luminodesmus* is continuous, but fluctuates by 20 to 40 per cent or more. The mechanism by which light emission is controlled is not known. No evidence was found for the suggestion that the light is bacterial in origin.

2. The luminescence is green with a maximum emission at 495 mμ and is optimal at a temperature of 31.5° C. Light emission is greatest in pure oxygen and extinguished in pure nitrogen. An oxygen concentration of 6.5% decreased the intensity from that in air by about one half.

3. Luminescence in water extracts of dried acetone powders has been demonstrated.

4. We have not been able to restore luminescence to dark extracts either by the classical luciferin-luciferase technique or by adding a variety of biochemical inter-

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[2] Substances tested for activity, either singly or in combination, were: adenosine triphosphate (ATP), $MgSO_4$, riboflavin, flavin mononucleotide (FMN), flavin adenine dinucleotide, oxidized and reduced diphosphopyridine nucleotide, oxidized and reduced triphosphopyridine nucleotide, coenzyme A, beef heart extract (Armour), yeast concentrate (Sigma), liver concentrate (Sigma), do-decyl aldehyde, ethyl alcohol, glycerol, glucose, glucose-1-phosphate, thiomalate, thiooctate, glutathione, cystine, cysteine, KCN, sodium arsenite, iodoacetate, sodium fluoride, sodium azide, p chloro-mercuro-benzoate, naphthoquinone, quinhydrone, hydroquinone, quinone, firefly extracts, NaCl, Na_2HPO_4, KH_2PO_4 and $MnSO_4$.

mediates. We have found that if adenosine triphosphate is added to extracts while they are luminescing an increase in light intensity occurs.

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THE ANTIMITOTIC AND CARCINOSTATIC ACTION OF OVARIAN EXTRACTS[1]

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The search for chemical substances which might have a retarding effect on the growth of tumors has led along many paths. All sorts of substances have been tried. Those investigators who have theorized at all have for the most part thought in terms of some block of metabolic activity. Our own program has sought to discover relatively non-toxic antimitotic substances of natural origin, and in the search for such substances we have based our attack on what we believe to be a proper theory for the initiation and suppression of mitosis. Such a theory is discussed in some detail in a recent book (Heilbrunn, 1956). It holds that the mitotic spindle results from a gelation of the protoplasm, the mitotic gelation. Various substances can prevent this gelation by keeping protoplasm fluid. And because the most usual type of protoplasmic gelation and the type involved in the mitotic gelation is a clotting similar to the clotting of blood, it is our belief that anticlotting agents such as heparin or similar substances can prevent cell division. This indeed they do. The protoplasmic colloid contains substances which favor clotting and those which tend to prevent it. We have made extracts from various tissues and have found that ovaries are especially rich in anticlotting agents. These appear to resemble heparin and to be mucopolysaccharides. This work (Heilbrunn, Wilson and Harding, 1951; Heilbrunn, Chaet, Dunn and Wilson, 1954; Heilbrunn and Wilson, 1956) showed that the ovaries of various invertebrates and fishes do actually contain antimitotic substances which prevent the mitotic gelation and suppress cell division. In the search for some substance or substances which might eventually prove to have clinical value, we have recently investigated the ovaries of mammals and especially large mammals. In what follows, we will attempt to show first that extracts of mammalian ovaries do have antimitotic action, an action which is associated with a liquefying or anticlotting effect on the protoplasm; and second that such extracts may possess carcinostatic activity.

MATERIALS AND METHODS

In studying antimitotic activity, the most favorable test objects and the easiest to work with are the eggs of various marine invertebrates. Eggs such as sea urchin eggs or those of the marine worm Chaetopterus divide synchronously following fertilization. These eggs can be obtained in large quantity and they represent a surprisingly constant material. In previous studies on Chaetopterus eggs, we have described the simple techniques required (Heilbrunn and Wilson, 1948). Most of

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our work was done on Chaetopterus eggs. One experiment was done on eggs of the clam Spisula. The technique required for the use of this egg has been described by Allen (1953). All of our experiments were done at a controlled temperature of 21° C. Two minutes after insemination the eggs were placed in the solutions to be tested.

Crude extracts were prepared by cutting the ovaries into thin slices and immersing these slices in solutions of acidified sea water at a pH of approximately 4.8. For each gram of tissue, one ml. of sea water was used. After extraction the extract was brought to the pH of sea water by the addition of NaOH. In a few cases the ovaries were homogenized before extraction, but this seemed to produce less favorable results in our antimitotic studies. The acidification of the sea water used in extraction was apparently not necessary and potent antimitotic extracts could be obtained over a wide range of pH values.

The Chaetopterus egg has a very fluid protoplasm which stiffens markedly before the formation of the mitotic spindle. There is indeed a two-fold increase in the viscosity of the protoplasm and this can readily be followed with the centrifuge method described in earlier studies. With a simple hand centrifuge, turned so as to give a force approximately 2250 times gravity, for some time after fertilization it requires only 7 seconds to move granules through the protoplasm of the Chaetopterus egg to such an extent as to give the appearance of zones. The number 7 is taken as a relative value for the viscosity of the protoplasm; actually it probably represents about twice the value of the viscosity in centipoises. At approximately 30 minutes after fertilization, 14 seconds of exposure to a force 2250 times gravity is necessary to produce the appearance of zones in the protoplasm. In studying the antimitotic and the anticlotting action of our extracts we determine the percentages of cleavage following exposure to various dilutions, and we also determined the effect of the extracts on the viscosity of the protoplasm at the time when the normal control eggs showed increased viscosity. These tests had to be made rapidly, for the duration of the mitotic gelation is short.

Crude extracts prepared in the way we have indicated are very potent anticlotting and antimitotic agents, as our results will show, but generally speaking they are not very effective as carcinostatic agents. Indeed these extracts contain not only substances which tend to prevent protoplasmic clotting, they also contain substances which have exactly the opposite effect. Thus, although in one case we did obtain a definite carcinostatic effect with such crude extracts, for the most part we were not successful, and indeed in some instances the survival time of cancerous mice treated with crude extracts was decreased rather than increased. Hence we were led to try and find methods of extraction which would give us preparations of as high a carcinostatic action as possible with a minimum of toxicity or adverse action.

For many months, we had little or no success. Then we hit on a method of fractional alcoholic precipitation, and this has now provided us with extracts which possess a definite carcinostatic action. This was perhaps to be expected, for in studying the antimitotic action of extracts of starfish ovaries, we found that the potent substance could be precipitated by alcohol (Heilbrunn, Wilson and Harding, 1951).

The procedure we finally adopted was the following: Cow ovaries, fresh from the slaughter-house, were ground up in a meat grinder and were then extracted in a solution containing 0.9% sodium chloride and 0.125% sodium bicarbonate. For each gram of ovarian material, 2 ml. of solution were used. The extraction was carried out in a cold room at 5° C. with constant stirring, and was continued for 16–18 hours. After the extract was strained through cheesecloth, it was centrifuged at 2000 rpm in a refrigerated centrifuge. The supernatant was then centrifuged in a Spinco centrifuge at 16,000 rpm (20,000 g) for an hour. The resultant supernatant was then precipitated by various concentrations of alcohol at approximately 0° C. Following each precipitation by a given percentage of alcohol, the supernatant was decanted. The precipitates were then lyophyllized. Our final product represented only a small fraction of the original ovarian material. Thus typically by precipitation with 45–60% ethanol we obtained about 10 mg. from a kilogram of ovaries.

In studying carcinostatic action, we used Swiss white mice. These were inoculated routinely with 1,500,000 Ehrlich ascites tumor cells in a volume of one ml. of ascitic fluid. Then 24 hours after inoculation, treatment with the extracts was begun. Each day for five days, each one of the mice to be treated was injected with a solution containing 15 mg. of the material. In general the 15 mg. were dissolved in 0.5 ml. of saline solution. Details of our technique and growth studies of our particular tumor will be published in a paper to be written by two of us (Tosteson and Davidson).

RESULTS

In the summer of 1955, we tested the effect of crude extracts of mammalian ovaries on cell division in the Chaetopterus egg. These extracts in every case stopped cell division. They also prevented the mitotic gelation. Our results are shown in Table I. In most cases the viscosity of the protoplasm at 30–40 minutes after fertilization is given as "less than 8." It may have been decidedly less than 8, but in the short space of time available for these measurements it was not possible to make enough tests to be sure of an exact value. However, the fact that the viscosity was less than 8 is a sure indication that the mitotic gelation has been suppressed, for during this gelation, the viscosity rises to a value of 14.

In addition to the data presented in the table, we have results from a few additional experiments; these results are entirely consistent with those shown in the table. In most of these other experiments, we attempted to improve the potency of the extracts by purifying them, but in every case the crude extracts prepared as described in the previous section were superior to the "purified" product. We also tried modifying our extraction procedures. In the results reported in the table, the extractions were made at a pH of 4.8, but we also tried extracting the ovarian material at pH's of 2.25, 3.90, 6.15, 7.1, and 10.0. All of the extracts prepared at these different pH's were likewise effective. Heating the extracts did not seem to have a very harmful effect, although our results were somewhat variable and minor differences in procedure seemed to be important. In one experiment an extract kept at 100° C. for three hours was still highly potent. In other experiments, exposure to 100° C. for shorter periods caused some loss in potency. Extracts made in distilled water and then lyophyllized were not effective. When the ovaries were homogenized before being extracted, the results were less favorable. Homogenization seemed to favor the release of thromboplastic substances into the extraction medium.

Although during the summer of 1955, we were never able to obtain any degree of successful purification of our extracts, in the following winter one of us (R. J. Rutman) hit upon the plan of precipitation with various concentrations of alcohol. Some of the precipitates obtained in this way when dissolved in saline solution had a very definite carcinostatic action, and we determined therefore to test the antimitotic action of these precipitates. Chaetopterus eggs (at Woods Hole) are only available during the summer months, so that we had to wait until the summer of 1956 before making our tests.

In making these tests, we had some difficulty, for the precipitates dissolved scarcely at all in the sea water in which we had to use them. However what tests we were able to make with the limited amount of material we had at our disposal indicated that only those precipitates obtained with intermediate concentrations of alcohol were effective. We had three fractions, of which A represented the precipitate obtained from cow ovary extracts with 0–45% alcohol, B the precipitate obtained with 45–60% alcohol, and C the precipitate with 60–80% alcohol. Of these three fractions, neither A nor C was very soluble. Fraction B seemed to go into solution, but when the solution was looked at under the microscope it was seen to have a large number of small solid particles suspended in it. We did several experiments with this fraction B. In one of these experiments, 5 mg. were dissolved in one ml. of sea water and then this solution was diluted so that the resultant dilutions contained 2.5 mg. per ml. of sea water and 1.25 mg. per ml. of sea water, respectively. All three of these solutions prevented the mitotic gelation and in all of them the fluidity of the protoplasm of the Chaetopterus eggs was maintained. In the most concentrated solution only 29% of the eggs cleaved, in the middle concentration 19%, and in the most dilute of the three solutions, 26% of the eggs cleaved. In the control 90% of the eggs cleaved. These counts were made 30 minutes or more after 50% of the control eggs had cleaved. The fact that the

TABLE I

Effect of ovarian extracts on the mitotic gelation and on cleavage of the eggs of Chaetopterus

Source of material	Duration of extraction in minutes	Dilution	Viscosity at 30–40 min.	% cleavage	% cleavage in control
Cow	30	—	4	1	98
Cow	60	—	4	0	98
Cow	120	—	less than 8	0	100
Cow	120	$\frac{1}{2}$	less than 8	0	100
Cow	120	$\frac{1}{4}$	about 8	0	100
Cow	120	$\frac{1}{8}$	about 8	5	100
Cow	60	—	less than 8	0	99
Cow*	60	—	5	0	99
Cow**	60	—	5	0	99
Cow	120	—	less than 8	0	99
Cow	120	—	less than 8	0	100
Pig	155	—	less than 8	0	99
Lamb	105	—	less than 8	0	99

* Ovarian capsules.

** Corpora lutea.

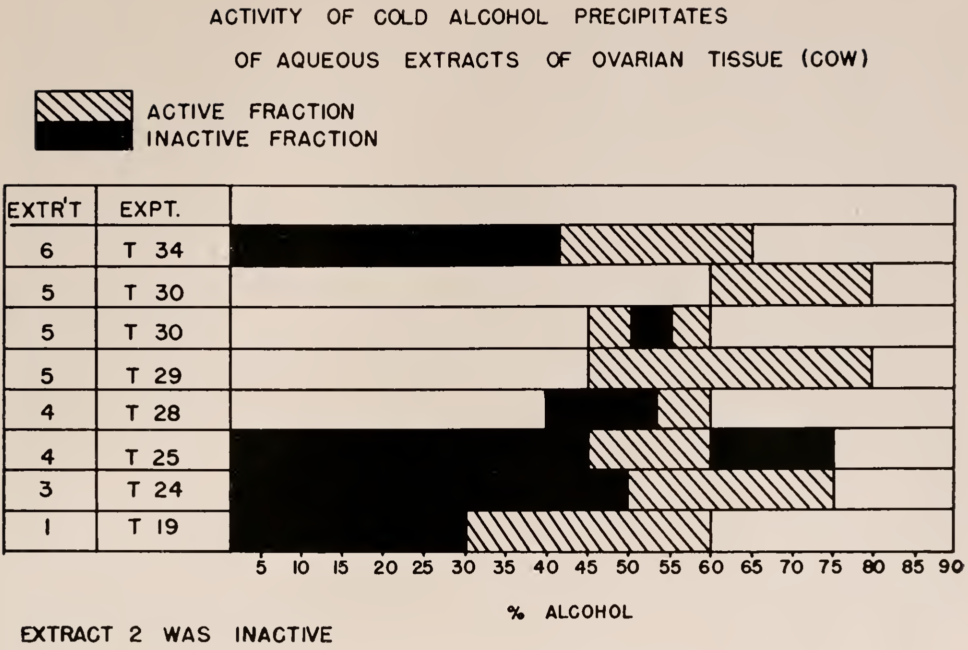

FIGURE 1. Activity of cold alcohol precipitates of aqueous extracts of ovarian tissue (cow).

various concentrations of material all acted in essentially the same way indicates that the material was only slightly soluble so that the actual concentration in true solution was the same in all three cases. In the very few experiments that we did with fractions A and C, these were without effect, perhaps because their solubility was extremely low.

In addition, the material of fraction B was tried on the eggs of the clam Spisula, and in this case also the material exerted an antimitotic effect. Concentrations of 2.5 mg. per ml., 1.25 mg. per ml. and 0.625 mg. per ml. showed strong antimitotic action, the cleavage being reduced from a control value of 97% to values of 31% for what presumably was the more concentrated solution, 39% for the intermediate concentration, and 40% for the weakest concentration.

Thus in spite of the fact that the material was soluble only slightly, the fraction obtained by precipitation with 45–60% alcohol did actually exert an antimitotic action, and in the case of Chaetopterus eggs this action was associated with a prevention of the mitotic gelation. No attempt was made to study the effect of fraction B on the mitotic gelation in the Spisula egg, for as yet the cycle of viscosity changes in the Spisula egg has not yet been worked out with sufficient thoroughness.

Clearly, our results with marine eggs indicate that fractionation of the crude cow ovary extracts by alcoholic precipitation can preserve the antimitotic and antigelating action.

Let us consider now the carcinostatic action of these alcoholic precipitates obtained from extracts of cow ovaries. Up until the present, we have accumulated a large body of data. Indeed we have experimented with well over 4,000 mice. Our results show that the precipitates obtained by treating cow ovary extracts with intermediate concentrations of alcohol can cause survival of some 15–25% of mice previously inoculated with a lethal tumor, a tumor which regularly kills 100% of all mice properly inoculated with it.

Figure 1 illustrates the fact that treatment with intermediate concentrations of alcohol in the cold can produce precipitates which have a carcinostatic action. This figure gives the results obtained with five different extracts and is based on experiments with about 2,000 mice. Further details of these experiments will be presented in another paper soon to be prepared by several members of our group (Tosteson, Davidson and Rutman). Since the data for the table were collected we have obtained additional confirmatory data. Also we have been experimenting with various other types of extraction media and with other types of fractionation. Preliminary results lead us to the hope that extracts and fractions can be obtained which will cause a higher percentage of survival than we have been able to obtain with the extracts described in this paper.

Discussion

Our work has been based on the idea that the protoplasm of all cells is much alike both chemically and physically, that inasmuch as the process of mitosis is much the same throughout the animal kingdom, the forces and agents which initiate cell division and those which suppress cell division are also much the same. On the basis of this pattern of thought, and on the basis also of many experiments on the initiation and suppression of mitosis (see Heilbrunn, 1956), we have been able to develop a new type of carcinostatic agent. At present this agent is at least as potent for our Ehrlich ascites tumors as other long-studied agents. It is our hope that the type of agent we are using can be perfected to give even better results. This will require much additional work.

Summary

Extracts of the ovaries of cows, pigs and sheep can suppress mitosis in eggs of the worm Chaetopterus. This they do by keeping the protoplasm fluid and inhibiting the mitotic gelation which is a necessary precursor of the mitotic spindle. The potent substance or substances in extracts of cow ovaries can be precipitated by treating the extracts in the cold with intermediate concentrations of alcohol. Such purified preparations have a definite antimitotic effect and they also have a very definite carcinostatic action.

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CARDIAC PHYSIOLOGY OF THE SCORPION PALAMNAEUS BENGALENSIS C. KOCH[1]

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The nature of heart-beat among arthropods has been studied by several workers (Prosser, 1942; Needham, 1950; Krijgsman, 1952). It has been observed in Crustacea and Insecta that the origin of heart-beat in each class is of varied types and has no relationship with the taxonomic classification. Among the arachnids, *Limulus* has a myogenic heart-beat in the young which becomes neurogenic in the adult (Prosser, 1942; Krijgsman, 1952) and the heart-beat of spiders is neurogenic (Rijilant, 1933). Even though Police (1902) indicated the presence of an epicardiac nerve on the heart of scorpion, the heart-beat of the scorpion, *Palamnaeus bengalensis* has been reported by Kanungo (1955) to be myogenic. It is of interest to find that, also in Arachnida as in Crustacea and Insecta, the nature of heart-beat is not of a single type. The hearts of arachnids are poorly understood and the present work is a detailed study of the physiology and pharmacology of the heart of *Palamnaeus bengalensis*.

Materials and Methods

Scorpions freshly collected from their natural habitat (Lal and Kanungo, 1953) were used for these experiments. They were lightly chloroformed and immediately dissected in a saline containing sodium chloride, 0.65 gm.; potassium chloride, 0.03 gm., and calcium chloride, 0.03 gm.; in 100 ml. of distilled water. The saline, which was prepared fresh before the experiments, was maintained at pH 6.3 using phosphate buffer, as the haemolymph of the scorpion was found to be on the acid side of neutrality in agreement with Maluf's (1939) statement. The heart was exposed fully *in situ* by carefully cutting the terga at the sides and removing them. Isolated heart preparations were made in petri dishes containing the saline. Effect of pH, temperature and drugs on the heart-beat were observed on hearts both *in situ* and isolated. Nearly 150 heart preparations have been made for various drug experiments.

Anatomy of the Heart

Like that of *Buthus* (Parker and Haswell, 1940), the heart of *P. bengalensis* is eight-chambered, spongy and muscular. It is enclosed in a pericardium and is held in its position between the two lobes of the liver by eight pairs of alary muscles. It is 2.5 cm. long in a medium-sized scorpion with a body length of approximately 5 cm. A thin-walled anterior aorta arising from the heart bifurcates on the oesophagus. A thin-walled posterior aorta proceeds to the tail.

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General Properties of the Heart-Beat

Hearts both *in situ* and isolated showed a high degree of automaticity; contractions occurred simultaneously throughout the myocardium and following one another in a regular uninterrupted sequence rhythmically. Simultaneous contraction of the scorpion heart had been reported earlier (Du Buisson, 1925). When first isolated, the rate of beat of the heart was irregular and slow, but it became normal after about 5 minutes, and showed a little acceleration in the rate of beat as compared with that of hearts *in situ*. The rate of beat of intact hearts was 50–54/minute at room temperature (25–27° C.). Cutting of the alary muscles *in situ* resulted in a slight increase of the rate to 56–62/minute, which was the same as that of isolated hearts. It appears, therefore, that even though the property of automatic movement lies in the muscles of the heart itself, the regulation of the rate of beat is effected by alary muscles. The rate of isolated heart preparations remained normal for 10–12 hours after which it decreased and the amplitude fell gradually. Mechanical stimuli like shaking the saline or pressing the heart with a needle temporarily inhibited the heart-beat. After 2–4 minutes, an acceleration in the rate was observed. Excised pieces of the heart beat for about 4–5 minutes. In all preparations, the anterior end stopped first and the posterior end later, suggesting that the pace-maker of the heart is situated at the latter end.

Course of Circulation

As stated above, isolated hearts beat with the anterior and posterior ends contracting and relaxing simultaneously. During diastole the heart shortens in length and bulges and the haemolymph flows in through the paired ostia. During systole, the heart extends lengthwise, the ostial valves close and the haemolymph is expelled at both the ends. A freshly isolated heart was placed in a dry watch glass in such a manner that the two ends were at a higher level than the middle region. A drop of neutral red was put at the center of the watch glass. The heart continued to beat and neutral red was seen flowing out at both the ends.

Effect of Temperature

Isolated hearts in petri dishes containing the saline were kept at different temperatures in an incubator and their rates were noted. An upper limiting rate of 80–85/minute was observed at 42° C., above which beating ceased permanently. The lower limiting rate on cooling was 4–5/minute at 5° C. Below this temperature the heart ceased beating but recovered when the temperature was increased.

Effect of pH

Separate stocks of the same saline solution were prepared by buffering with phosphate buffer between pH 5.5 and pH 7.5 and the hearts were kept in these salines. The heart remained active between pH 6.1 and pH 6.5. With increase or decrease of the pH of the saline beyond this range, depression of the heart rate occurred.

Effects of Drugs

Fresh dilutions of 10^{-3}, 10^{-4}, 5×10^{-4}, 10^{-5}, 5×10^{-5}, and 10^{-6} of various drugs were made in the saline before each set of experiments. Both intact and isolated hearts were bathed side by side with one of the diluted drugs to compare their effects on the heart-beat in isolated and *in situ* preparations. No difference between isolated and intact hearts was observed. Mechanical shock to the heart was avoided as far as possible. The drug was sucked out with a pipette, the heart washed with the saline three times and the fresh drug added slowly. Recordings of the heart rate were made following the methods of Jones (1954). Three recordings, one minute each in length, were made one minute after adding the drug.

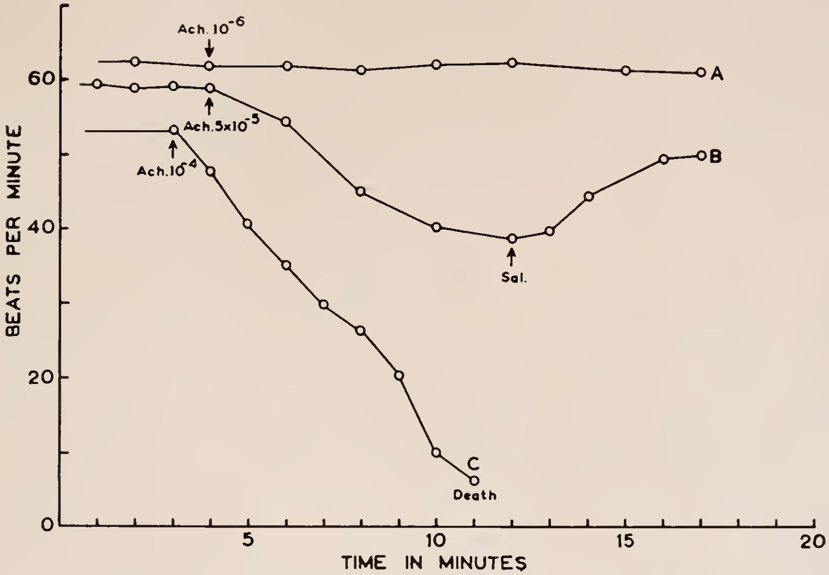

Figure 1. Effect of acetylcholine on the heart of *P. bengalensis*. Ach., acetylcholine; Sal., saline.

Acetylcholine more dilute than 5×10^{-5} had no effect on the heart rate. Concentrations of 5×10^{-5} or stronger depressed the heart rate; the time taken for depression was inversely proportional to the concentration of the drug (Fig. 1). There was a gradual weakening of the strength of beat, reduction in the amplitude, rest-pauses and sporadic irregularities in 5×10^{-5} or stronger concentrations. In no case was there any acceleration before the depression. Neither did the beat recover if the heart was left in the drug. However, all such hearts recovered after they were washed with the saline, but the normal rate of beat was never reached. Some hearts showed tolerance to the drug up to 10^{-4} after prior treatment with more dilute solutions and gradually increasing concentrations (Fig. 2D).

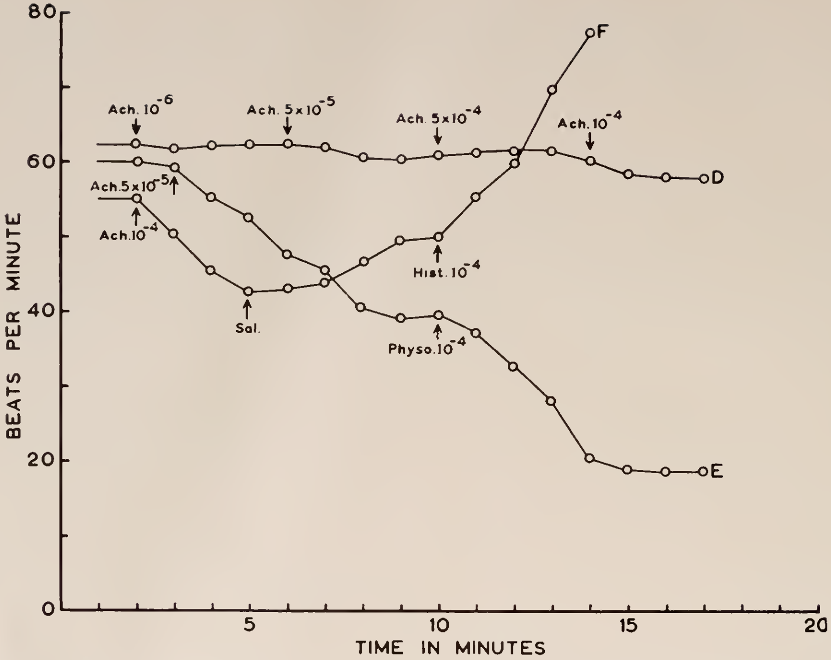

FIGURE 2. Effects of various drugs on the heart of *P. bengalensis*. Ach., acetylcholine; Adr., adrenaline; Atr., atropine; Hist., histamine; Physo., physostigmine; Sal., saline.

Physostigmine at 10^{-4} or stronger did not by itself show any appreciable effect on the heart rate. However, application of acetylcholine to the heart, after treatment with physostigmine, potentiated the effect of acetylcholine (Fig. 2E).

Histamine at 10^{-4} or stronger accelerated the heart rate to a maximum of 85/minute and this effect was reversible on washing with the saline. The time taken for the heart to reach the maximal rate in different dilutions was directly proportional to the dilutions of the drug. It antagonized acetylcholine action, and hearts collapsing under the treatment with acetylcholine could be revived by this drug. Such hearts also beat at 85/minute (Fig. 2F).

Adrenaline at 10^{-5} or stronger accelerated the heart rate to about 75/minute and this effect was reversible.

TABLE I

Comparison of the effects of drugs on the hearts of Limulus and P. bengalensis

	Ach.	Atropine	Adrenaline	Ether	Histamine	Physostig-mine	Chloroform
*Limulus**	+	+	+				
P. bengalensis	−	−	+	0	+	0	0

+, excitation; −, inhibition; 0, no effect.
* Krijgsman, 1952.

Atropine at 5×10^{-4} or stronger inhibited the heart rate reversibly.

Half-saturated and fully-saturated aqueous solutions of *ether* had no observed effect on the heart-beat.

Chloroform had no observed effect on the heart-beat.

It was found in all the cases that the drug-treated hearts recovered after washing with the saline. A quicker recovery of the heart was attained by using warm saline which was added slowly to the heart container. The time taken for such recovery varied from five to fifteen minutes.

Table I gives comparatively the effects of various drugs on the hearts of *Limulus* and *P. bengalensis.* Even though Table I does not indicate the effect of ether on the neurogenic heart of *Limulus,* it may be mentioned here that ether inhibits neurogenic hearts in low concentrations (Needham, 1950).

Haemolymph Pressure

Bleeding occurred when incisions were made at pedipalpi, abdomen and tail regions; this indicates positive haemolymph pressure throughout the body. By inserting capillaries in continuation with U-tubes, actual pressure was found to be 6 mm. of saline at the pedipalpi and at the abdomen.

Discussion

The pharmacology of the scorpion heart resembles that of the crustacean, *Daphnia* (Baylor, 1942) and the vertebrates, and has no resemblance to that of *Limulus.* According to Prosser *et al.* (1950), the hearts of arthropods are of non-innervated myogenic, innervated myogenic and neurogenic types, which description is based mainly on acetylcholine effect. Needham (1950) classified the crustacean hearts into two categories, myogenic and neurogenic, by taking several factors into consideration. The heart of *P. bengalensis* in showing (1) autonomous rhythmicity with contractions developing simultaneously throughout the myocardium, (2) insensitiveness to ether, and (3) inhibition by acetylcholine indicates that the nature of its beat or that of its pacemaker is innervated myogenic. The epicardiac nerve reported by Police (1902) appears to be either extrinsic or regulating in function.

Summary

1. The heart of *P. bengalensis* beats continuously at a rate of 50–62/minute at a temperature of 26° C. The contraction is developed simultaneously throughout the muscle.

2. Acetylcholine and atropine depress the heart-beat and their actions are reversible. Physostigmine potentiates the effect of acetylcholine.

3. Ether and chloroform have no effect on the heart-beat.

4. Histamine and adrenaline accelerate the heart-beat and their effects are reversible on washing with saline.

5. The haemolymph pressure is 6 mm. of saline.

6. It is concluded that the pace-maker of the heart of *P. bengalensis* is of the innervated myogenic type.

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A COMPARATIVE STUDY OF THE CUTICULAR STRUCTURE OF THREE FEMALE MEALY BUGS (HOMOPTERA: PSEUDOCOCCIDAE)

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The recent discovery in the arid north-west of South Australia of a new species of the hitherto monotypic genus, *Epicoccus,* afforded an opportunity to examine the cuticle of a drought-resistant pseudococcid. Members of the Pseudococcidae are normally confined to microhabitats where they are surrounded by humid equable conditions, and it was anticipated that considerable modification of cuticular structure would be shown by a species which has evolved in an area where it is fully exposed to the desiccating effects of high temperatures, low relative humidities, and drying winds. The extent of such modification, if any, could be gaged only after the cuticle had been compared with that of a typical form, of which, however, there appears to be no published account. A study of the cosmopolitan long-tailed mealy bug, *Pseudococcus adonidum* L., was therefore undertaken as a preliminary step in the investigation. Finally, the cuticle of the one described species of Epicoccus, *E. acaciae* (Maskell) was examined to ascertain whether any major differences in cuticular structure occur within the genus.

The terminology is in accord with the scheme which I recently outlined (Lower, 1956).

Materials and Methods

Specimens of *P. adonidum* were obtained locally from a heavily-infested plant of *Daphne odora.* Those of *Epicoccus* sp., were collected from *Acacia aneura* F. Muell., at Yudnapinna in the north-west of South Australia while those of *E. acaciae* came from the coastal strip of Western Australia, the only area in which the species is known to exist.

The insects were first killed with cyanide, and free-hand sections of some of each species at once stained with Sudan black B. The remainder, after fixation in Sanfelice's fluid, were embedded, part in a water-soluble wax and part in paraffin. Sections cut from these were similarly stained. Comparison showed that there was no observable loss of cuticular lipoid when paraffin was used as the embedding medium. All work was therefore done using paraffin sections cut at 4 μ and 1 μ, except that when the external wax of *Pseudococcus* was being investigated, sections prepared by the first two methods were used.

The histochemical tests and techniques applied have been described elsewhere (Lower, 1957a).

I. The Cuticle of Pseudococcus adonidum L.

The cuticle (Fig. 1) is thin, measuring in most parts between 6 μ and 8 μ; only exceptionally does it attain a thickness of 10 μ. Its structure is relatively un-specialized and except for the brown outer layer of the epicuticle it is unpigmented. Pore canals, if present, could not be observed with the light microscope either under bright-field or phase-contrast conditions. A thick layer of wax (discussed later) covers the cuticular surface.

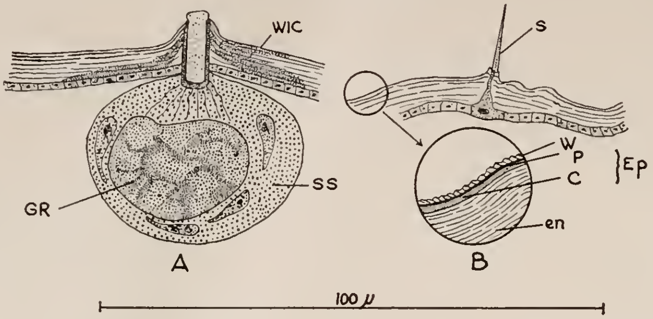

FIGURE 1. *P. adonidum:* Cuticle. A. Part of cuticle adjoining glandular duct. B. General cuticle. c, cuticulin layer; en, endocuticle; Ep, epicuticle; GR, gland reservoir; P, paraffin layer; S, spinule; SS, secretory sheath; W, surface wax layer; WIC, wax-impregnated cuticle.

A. The Epicuticle

The epicuticle (Fig. 1B) is two-layered and has a total thickness of about 1 μ, of which the inner layer constitutes the greater part.

The inner or cuticulin layer is colorless and transparent. It responds positively to the Millon, xanthoproteic and biuret tests, is non-argentaffin, and is unaffected by either Sudan black B or Nile blue sulfate. It is stained red by the routine stain and pink by Sevki's diluted Giemsa technique, but it cannot be stained either by Mallory's PTAH or any of the iron haematoxylins. Schmorl's test shows the absence of reducing substances. It is soluble in warm concentrated solutions of potassium hydroxide or hydrochloric acid. Its location and general chemical reactions indicate that it is probably homologous with the cuticulin layer of *Rhodnius* (Wigglesworth, 1947).

The thinner outer layer is brown-pigmented. The color is difficult to discharge, sections requiring about a week's immersion in 10% hydrogen peroxide to effect this. The strong positive response to Schmorl's test, together with the non-argentaffin nature of the layer, suggests that the coloring matter is a lipofuscin. The layer responds to none of the protein tests. It is blackened by Sudan black B, and stained deep blue by Cain's Nile blue sulfate technique. Prolonged differentiation during the latter process removes much of the blue without any red appearing so that acidic lipoid only appears to be present. It is stained black by iron haematoxylins and deep violet by Mallory's PTAH. It is resistant to concentrated solutions of potassium hydroxide and hydrochloric acid but fuming nitric acid or *aqua regia*, when gently warmed, attacks some of its constituents and liberates a material which is practically instantaneously soluble in cyclohexane and is quickly and easily stained by any of the fat stains. Its anatomical position and general chemical behavior indicate that the layer is a paraffin epicuticle (Dennell and Malek, 1955). It differs from the corresponding layer in Sarcophaga (Dennell,

1946) by being non-argentaffin, in giving no response to the xanthoproteic reaction, and in its content of lipofuscin pigment.

B. The Procuticle

Practically the whole of the procuticle is present as endocuticle. With the exception of the spinules mentioned below, no exocuticle occurs, while the mesocuticle is confined to the alveoli of the spinules and the small convex circular patches (appearing crescentic in transverse section) where muscles are inserted in the cuticle.

Scattered over the cuticular surface are weakly-sclerotized, colorless spinules (Fig. 1B), each secreted by a trichogenic cell which is much larger and more granular than the adjacent hypodermal cells. A cytoplasmic process from each trichogenic cell, after passing through the cuticle, attenuates and forms a core to the spinule for its basal two-thirds. Both spinules and alveoli are feebly argentaffin and are stained by the routine stain, the former a very pale pink and the latter red.

The endocuticle is laminated and closely resembles that found in other insects with soft cuticles. Its principal constituent is the normal chitin-protein complex.

II. The Cuticle of Epicoccus sp.

To comprehend the structure of the cuticle of *Epicoccus*, some knowledge of its mode of development is essential.

At the end of a short period of wandering, the second female nymphal instar assumes a permanent position on a twig, petiole, or leaf, moults, and enters her third and final stadium. Rapid development of the cuticle now begins. The cells of the dorsal hypodermis hypertrophy and their secretory activity correspondingly increases. This gives rise to enormous allometric growth of the dorsal cuticle as two lobes, one along either side, whose inner surfaces are in close contact with the bark of the host. Posteriorly these do not clasp the stem but grow round until their margins meet enclosing a small orifice (Fig. 3). While these changes have been in progress, glands located along the parts of the lobes contiguous with the bark have been pouring out large quantities of wax, not unlike bees-wax in colour and texture, which cements the insect firmly to the surface of the stem. Meanwhile, its ventral surface has moved outwards away from the bark, the space so formed constituting a brood chamber (Figs. 2 and 3) whose only means of communication with the exterior is through the pore between the adpressed posterior tips of the lobes. As a result of these changes, the greater part of the cuticle, and the only part visible when the insect is *in situ*, consists of the large thickened dorsum. The remaining much-smaller enclosed region comprises the small thin sternal cuticle and the relatively-minute, degenerate pleural regions. The whole development resembles much more closely that of a coccid than that of a pseudococcid. The thickened dorsum, deeply infolded to expose the least possible area to the atmosphere, the enclosure of the entire thin part of the cuticle, and the spiracles opening into the brood chamber, all appear to be adaptations to minimize water loss.

The fully-developed female (Fig. 4) has an average length of about 3 mm. and is somewhat less than this in width. Transverse sections display a great variety of contour, the only consistent feature being their distorted U-shaped outline. Although an occasional isolated female may be almost bilaterally symmetrical, the normal condition, brought about by the gregarious mode of life, is for one or the

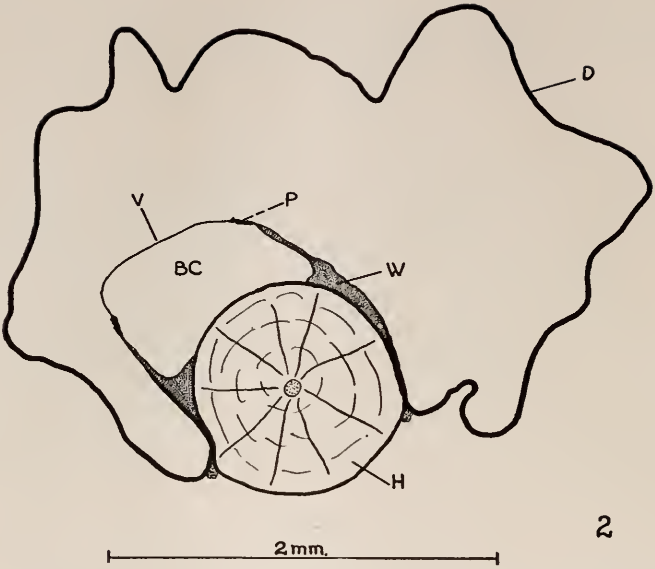

FIGURE 2. *Epicoccus* sp. Transverse section showing spatial relation between insect and host. BC, brood chamber; D, dorsal cuticle; H, host; P, pleural cuticle; V, ventral cuticle; W, cementing wax.

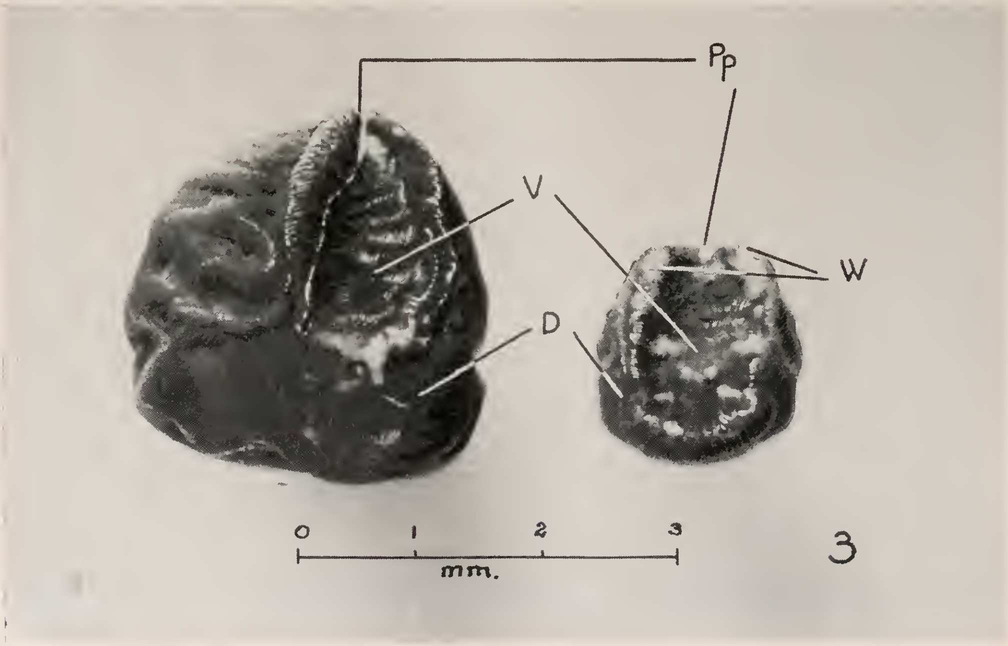

FIGURE 3. *Epicoccus* sp. Fully developed and immature third female instars. Ventral aspect showing brood chamber and posterior pore. D, dorsal cuticle; Pp, posterior pore; V, ventral cuticle; W, cementing wax.

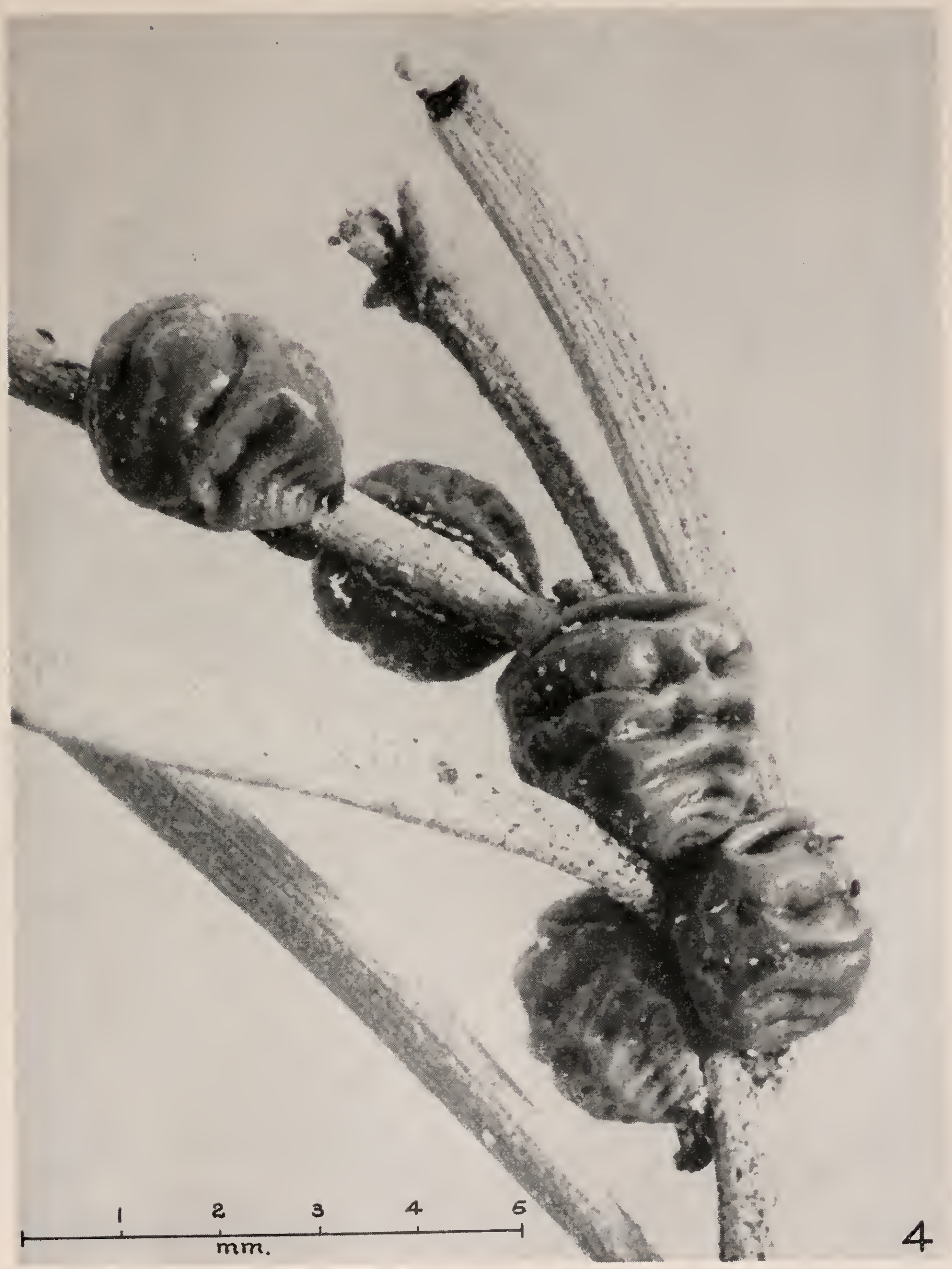

Figure 4. *Epicoccus* sp., *in situ. Photograph courtesy of Helen M. Brookes.* The females are invariably so oriented that the rounded cephalic end is directed towards the host's region of growth. Part of the cementing wax is visible on the second insect from the top.

other lateral lobe to be more developed than its fellow. Which of these is the larger is determined by the proximity of neighboring females, leaf petioles or similar obstructions, and the natural asperities of the bark encountered during growth. Figure 2 was drawn from a section selected, not because it was typical, but because it clearly displayed the spatial relation between insect and host.

The mouthparts excepted, the only regions of the body capable even of limited movement are the pleura and the ventral abdominal surface, both of which are subordinate to the functions of oviposition and defaecation.

The cuticle exhibits great diversity of thickness, structure and composition. For descriptive purposes, four major types are here recognized: the respective cuticles of the dorsum, the venter, the pleura, and the intersegmental membranes. The latter are restricted to the enclosed ventral surface; externally, they are suppressed by fusion of the segments and are represented only by sutures.

1. *The Dorsal Cuticle*

Relative to the size of the insect, the dorsal cuticle is massive, ranging in thickness from a minimum of 30 μ near its junction with the pleura (Figs. 5 and 6A) to some 40 μ in the remainder (Fig. 6C). Maximum thickness is attained in small localized patches where muscles, particularly those in the abdomen associated with the organs of oviposition, are inserted. In such areas thickenings of 70 μ or more are not uncommon (Fig. 6B). It exhibits a general uniformity of structure throughout, consisting of a two-layered epicuticle overlying the procuticle. It is interesting to note that no part of the cuticle is argentaffin.

A. The Epicuticle

Irrespective of its location, the epicuticle displays a constancy of thickness, structure, and composition so that what is said of it here in connexion with the

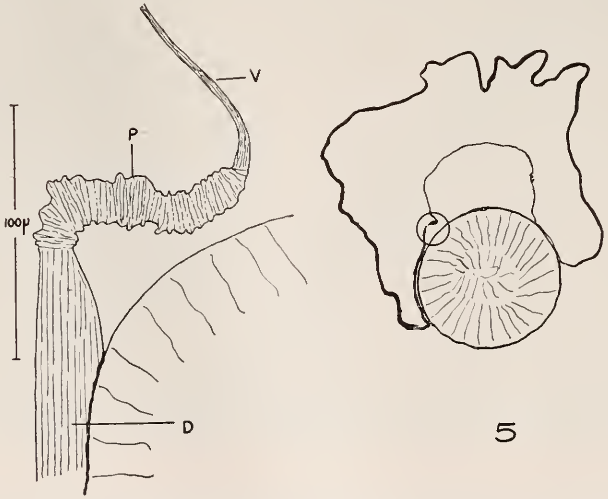

FIGURE 5. *Epicoccus* sp. Dorso-pleural and ventro-pleural junctions. The part of the cuticle drawn is indicated by the small circle in the diagram at right. Symbols as for Figure 2.

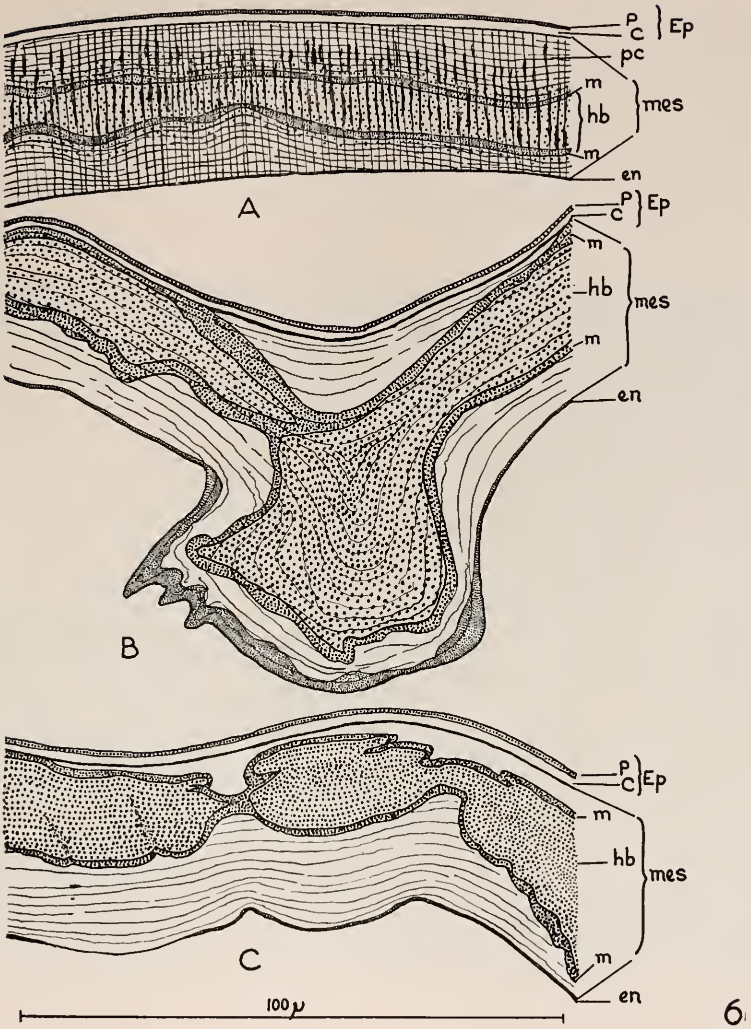

FIGURE 6. *Epicoccus* sp. Dorsal cuticle. A and C, general cuticle; B, cuticular ingrowth. Pore canals are omitted from B and C for clarity. hb, heavily-staining zone; m, margins of heavily-staining zone; mes, mesocuticle. Other symbols as for Figure 1.

dorsal cuticle has general application. Except for the non-pigmented condition of the paraffin layer, it is practically indistinguishable from the same structure in *Pseudococcus*. It has a total thickness of about 1.5 µ distributed between the (outer) paraffin component which constitutes about one third of it, and the (inner) cuticulin layer which comprises the remainder (Figs. 6 and 7).

The Cuticulin Layer:

The cuticulin layer is colorless and transparent. It gives a vigorous response to Millon's reagent, is stained yellow by the xanthoproteic reaction, and orange-red by the routine stain. It is non-argentaffin, non-reducing, non-iodophil, and is unaffected by Danielli's, Gibb's, or Mallory's PTAH techniques. It gives negative responses to Sudan black B and Nile blue sulfate. It is easily soluble in hot solutions of potassium hydroxide but resists for at least twelve hours the action of 10% hydrochloric acid at 60° C. These responses indicate a composition largely proteinaceous. The materials to which the protein is bound are such that the usual range of tests do not serve for their identification. They also confer on it its resistance to extraction by hot dilute acids.

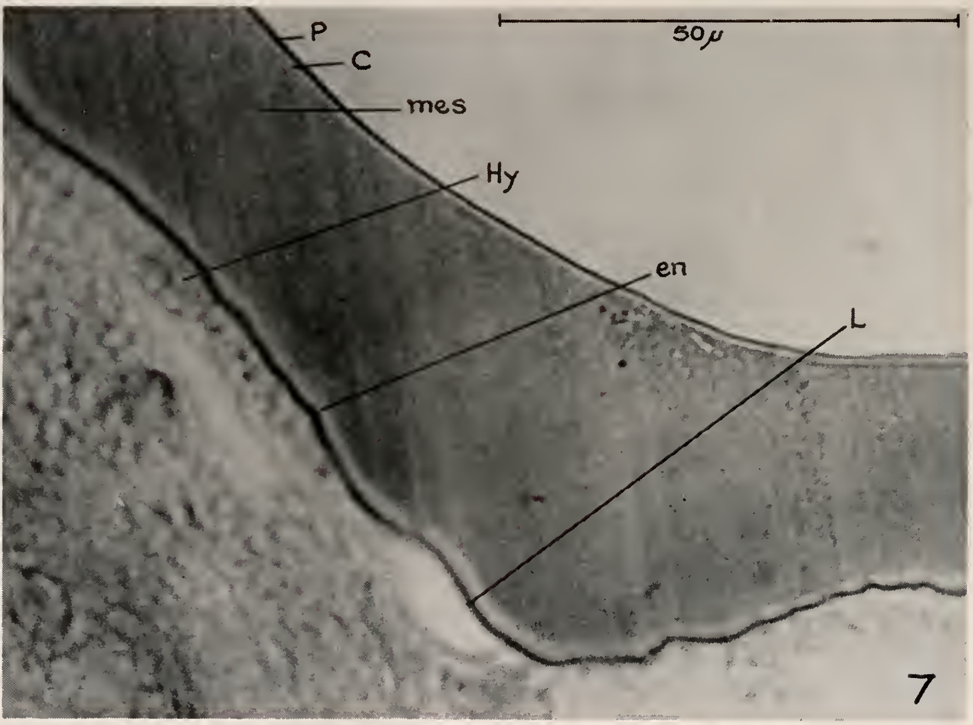

Figure 7. *Epicoccus* sp. Dorsal cuticle 4 μ; stained with Sudan black B, bright-field. Hy, hypodermis; L, lipoid zone; mes, mesocuticle. Other symbols as for Figure 1.

The Paraffin Layer:

This layer is colorless. Its characteristic component is lipoid, as the strong positive responses to fat stains (Fig. 7) and osmic acid indicate. Schmorl's test shows it to possess strong reducing properties and Mallory's PTAH stains it deep violet. It is intensely iodophil but it is not affected by the other reagents used nor is it soluble in dilute acids or alkalis.

The Surface Secretion:

The dorsal cuticle of living insects or of those killed with cyanide has a resinous luster (Fig. 4) which is retained when the dead specimens are dehydrated. The glossy surface cannot be wetted. If freshly killed or dried specimens are treated with fat solvents, 90% ethanol, or warm 5% sodium or potassium hydroxide solution for five minutes, and then washed with water and dried, the luster is lost and the entire surface becomes dull. Treatment with boiling water, hot concentrated hydrochloric acid, or exposure to dry heat at 100° C. for twelve hours does not affect it. Immersion of insects in molten water-soluble wax similarly leaves the luster undimmed.

Untreated and "dulled" insects were therefore embedded in this medium, the sections stained with aqueous dyes in acid solution, and mounted in glycerol which is without effect on the luster. Sections cut from both batches were indistinguishable nor did comparison with sections of fresh material reveal any recognizable differences.

This scanty evidence suggests that a layer of sub-microscopic thickness (possibly containing lipoid) may cover the paraffin layer of the dorsal epicuticle.

B. The Procuticle

The procuticle consists almost entirely of mesocuticle (Fig. 7). Exocuticle is restricted to small widely-dispersed papillae, each bearing a minute hemi-sclerotized seta. The endocuticle forms a narrow zone about 1 μ in thickness except at the termination of cuticular ingrowths where its lobes and thickened portions serve as intermediaries for muscle attachment (Figs. 6 and 7).

The Exocuticle:

In the sense that exocuticle is completely-sclerotized procuticle, this zone is wanting in *Epicoccus*. Both papillae and setules are transparent; the former are very pale yellow, the latter are colourless. Neither is inert to aniline stains as is true exocuticle. The routine stain colours both of them pink and the colour deepens as time of immersion is increased. Treatment for a few minutes with "Diaphanol" is sufficient to destroy the incipient sclerotization and they then stain intensely and rapidly with acid dyes. Their development appears to be in a stage intermediate between mesocuticle and exocuticle. Complete sclerotization commonly induces changes in the overlying cuticulin layer but the epicuticle of the papillae differs in no respect from that of other parts of the body.

The Mesocuticle:

The mesocuticle shows little structural differentiation. When unstained sections are examined under the highest powers of the light microscope the mesocuticle appears uniform and featureless. Under phase-contrast conditions it is transversely marked with numerous, irregular dark streaks which indicate the positions of pore canals of whose organization, however, no details are observable. The routine stain dyes the zone red but supplies little information on canal structure,

Chemically, the mesocuticle is as complex as it is structurally simple. Millon's reagent differentiates it into three clearly-defined sub-zones which, beginning with the outermost, are here referred to as the A, B, and C sub-zones, respectively (Fig. 8).

The A sub-zone is stained pink by Millon's reagent, and the distal parts of the pore canals appear as indistinct, thin red lines which, after traversing the region, attenuate before they terminate at its outer surface. It is of variable width; in some parts it may form as much as a third of the cuticle but is generally less. Occasionally it is suppressed by the outward extension of the B sub-zone (Fig. 6B).

The B sub-zone takes the form of a conspicuous, broad, bright-red band, sharply demarcated externally and internally from the remainder of the procuticle by narrow crimson margins (Figs. 6 and 8). It is continuous throughout the dorsal mesocuticle, though it thins before it terminates at the dorso-pleural junctions. This intensely-staining proteinaceous region exhibits great and sudden variations in thickness. Its maximum development may be observed in cuticular ingrowths of which it occupies the greater part (Fig. 6B). Where it is thinnest (Fig. 6C), it is composed solely of the contiguous crimson margins. The distinct differences in color between the central part and its marginal bands suggest either that different proteins are present in each or that the concentration of the one protein is higher in the margins than internally. The general appearance is reminiscent of the result obtained by the use of the paper-chromatography technique. The great mass of the pore canals (Fig. 9) is located within the zone and it is possible that a protein complex in solution diffuses from them into the circumjacent cuticle there to undergo partial separation, the chitinous matrix acting similarly to the chromatographic paper.

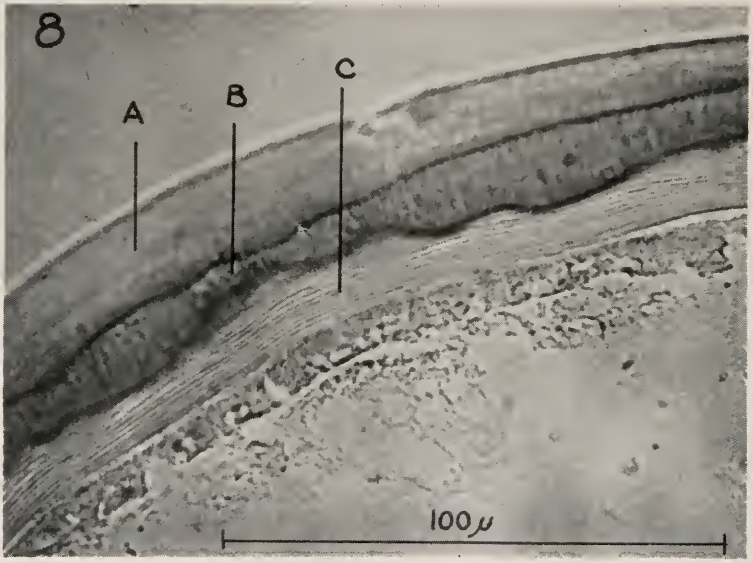

FIGURE 8. *Epicoccus* sp. Dorsal cuticle, 4 μ. Millon's reagent; bright field. A, B, and C are the three sub-zones referred to in text. Maximum definition in the mesocuticle has been sought; the epicuticle is distorted through being out of focus.

Sub-zone C is stratified in alternate red and pale-pink layers (Fig. 8). Whether or not these coincide with the probable original lamellate deposition of the procuticle cannot be determined since no other stain or technique used, produced comparable differentiation. This sub-zone varies in thickness but occupies about the inner third of the mesocuticle.

Of the wide range of tests applied to the mesocuticle none so clearly distinguished the sub-zones as did Millon's reagent. Other tests which responded positively gave diffuse results, but confirmed the fact that the concentration of protein in the B sub-zone is higher than in any other part of the mesocuticle. Ninhydrin colored the mesocuticle violet-pink, the greatest depth of color being developed in the B sub-zone. The xanthoproteic reaction stained it deep orange medially, paling to yellow towards either surface. The iodine technique (Lower, 1957b) approximately delineated the sub-zone by staining it deep purple-black, the C sub-zone was uniformly dark red, and the A sub-zone was practically unstained. Mallory's PTAH produced a similar picture, the B sub-zone being reddish violet with deeper violet margins, the C sub-zone light red, and the A sub-zone almost colorless.

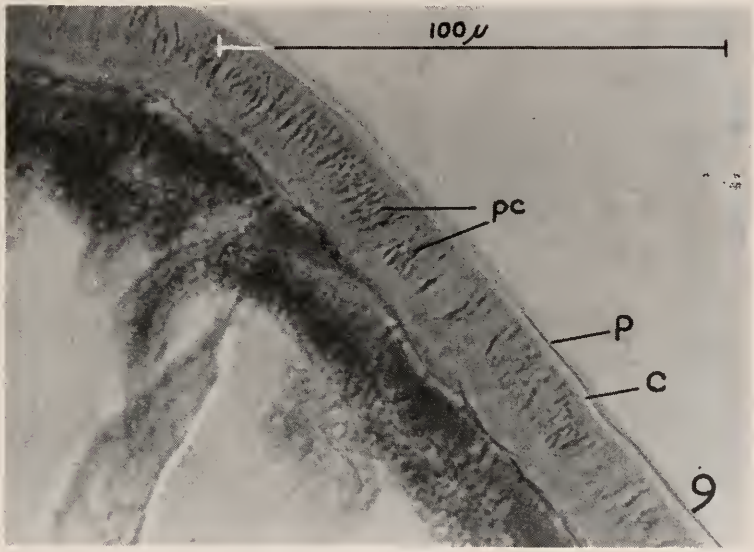

FIGURE 9. *Epicoccus* sp. Pore canals in latero-dorsal cuticle. One micron; stained by Sevki's technique; bright-field. Note termination of pore canals at outer mesocuticular surface. C, cuticulin layer; P, paraffin layer; pc, pore canals.

As is usually the case, Sevki's technique produced the clearest differentiation of the pore canals (Figs. 9, 10). Their thin hypodermal connections were colored red, their thickened portions in the B sub-zone were deep purple, and their thin terminal parts in the A sub-zone were red. Their numerosity is such that even in sections cut at 1 µ they appear as a confused mass (Fig. 10). The only valid conclusions that can be drawn concerning them are the following: the pore canals are confined to the procuticle, being continuous between its outer surface and the hypodermis; they are extremely numerous, of highly irregular form, and the mass of their contents, which possess a high concentration of tyrosine-containing protein, is almost entirely located within the B sub-zone.

The mesocuticular protein is separable into two fractions. Sections were immersed for twelve hours in 10% hydrochloric acid maintained at 60° C. After washing in distilled water it was found that the mesocuticle had lost its characteristic staining properties. Millon's reagent colored it uniformly pink and the xanthroproteic reaction pale yellow. Ninhydrin, Mallory's PTAH, and the Sevki and iodine techniques all gave negative results. The routine stain dyed it light red. In no section were pore canals visible and even the use of phase-contrast failed to reveal their positions. These results demonstrate that while most of the protein is extractable with hot dilute hydrochloric acid, there is a residual fraction firmly bound to the chitin in such a manner that its extraction is more difficult.

There is no evidence to suggest either the mode of accumulation or functioning of the apparently high protein concentration in the B sub-zone. The adult male of either species of *Epicoccus* is unknown; the cuticles of first and second nymphal instars of either sex do not differ significantly from those of the corresponding instars of other pseudococcids. Of the known stages, therefore, the phenomenon is confined to the third female instar in which the thickness of the sub-zone relative to that of the cuticle as a whole increases with the age of the insect.

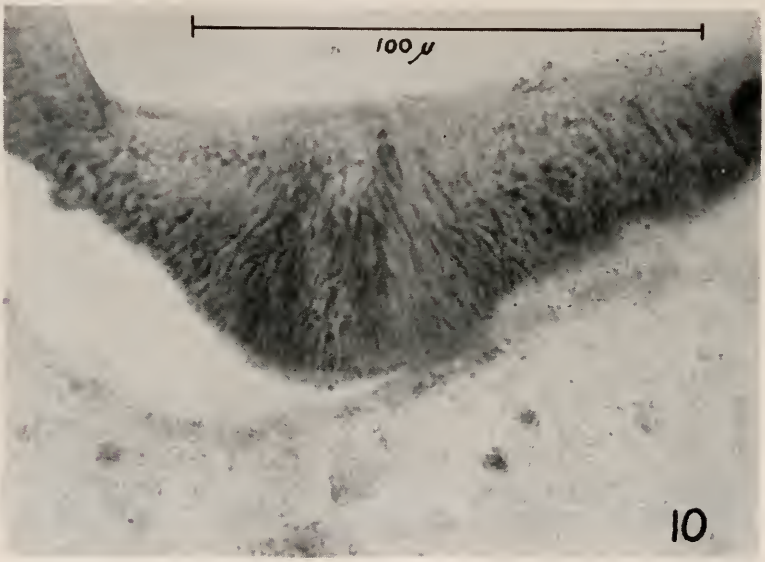

FIGURE 10. *Epicoccus* sp. Pore canals in thick part of dorsal mesocuticle. One micron; stained by Sevki's technique; bright field.

The Endocuticle:

As mentioned above, the endocuticle is greatly reduced. It is wanting in many parts of the cuticle, and, where present, is never more than 1 μ in thickness except where it caps the cuticular ingrowths. Its structure appears normal.

The Lipoid Zone:

When the routine stain is applied to a cuticular section, a thin layer, staining a much deeper green than does the endocuticle, can be seen to separate the latter from the hypodermal cells. This layer has an average thickness dorsally of about 1 μ, and is continuous throughout the dorsal cuticle except where interrupted by cuticular ingrowths. Laterally, it attenuates until it disappears in those parts of the dorsal lobes which are in contact with the bark. It is absent from the cuticles of the pleura, venter, and intersegmental membranes. In unstained sections, the layer is indistinguishable from the endocuticle, since the structure and natural color of both are the same. Its high content of chitin shows it to be definitely of procuticular origin, and but for its impregnation with other materials, it would merely represent the innermost part of the endocuticle.

It is remarkable for its high lipoid content, and to obviate additions to the terminology, unjustifiable at this stage, it is referred to descriptively as the *lipoid zone*. The lipoid zone has unusual chemical properties for a part of the procuticle, so anatomically located. In addition to the reactions characteristic of endocuticle generally, it is stained deep violet by Sevki's technique and Mallory's PTAH. It is most intensely colored, however, by the fat stains.

In free-hand sections of fresh material, the zone can be deeply stained at room temperature by short immersion in the fat stains. Sections cut from paraffin-embedded material, or those cut from cuticles which have been repeatedly extracted with boiling cyclohexane, cannot be stained in this manner. Nearly as intense color, however, can be developed by immersion for several hours at 60° C. in the same stains. The Nile blue sulfate technique shows that part, at least, of the lipoid is neutral, since the zone is stained red after differentiation. If sections cut from extracted or paraffin-embedded material be gently warmed with fuming nitric acid, the chitin-lipoid association is destroyed, and the previously-dispersed lipoid aggregates into minute droplets which stain rapidly and intensely with fat stains. These results indicate that a lipoid complex, rather than a single lipoid, is involved. Part of the lipoid is free, and easily extractable; part is bound to the chitin and the other constituents of the procuticle of the region, and resists extraction.

Of the origin and function of the lipoid zone, nothing is known. Examination of females in various developmental stages shows that lipoid impregnation synchronizes with secretion of the procuticle, and that the zone maintains its position relative to the hypodermis throughout the development. It is not present in the first and second nymphal instars, and was absent from one very young third female instar which had reached this stage only about the time that the insects were collected. In all the other females examined, no measurable difference in its thickness was observed, and in two exceptionally large specimens (both parasitized), the zone was of normal thickness.

Only twice previously has the presence of a layer between the endocuticle and the hypodermis been recorded in the literature. The two records are those of Schmidt (1956) and Malek (1956).

Schmidt reported that, in certain insects which he had examined, a glycoprotein layer, the "sub-cuticle," separated the endocuticle from the hypodermis. As he stated categorically that the "sub-cuticle" was non-chitinous, it clearly has no affinity with the lipoid zone of *Epicoccus,* and need not be further discussed here.

Malek demonstrated that when the desert locust, *Schistocerca gregaria,* is moulting, the inner part of what was originally endocuticle becomes impregnated with a lipo-protein complex, to form the ecdysial membrane of the insect.

This membrane and the lipoid zone of *Epicoccus* appear to be homologous structures. Since both are derived from the innermost part of the procuticle contiguous with the hypodermis, both occupy corresponding anatomical positions. Both are impregnated with lipoid, and both, by reason of their derivation, contain chitin.

The stages of the life cycle in which each is present are, however, completely reversed. In *Schistocerca*, the lipoid-impregnated procuticle must, by forming an ecdysial membrane, be necessarily confined to immature stages, since the adult does not moult. The lipoid zone of *Epicoccus*, on the contrary, is found in the adult only. Few details are given in Malek's preliminary note, and it will be necessary to await his full account, before an adequate comparison of the two structures can be made.

2. *The Ventral Cuticle*

The cuticle of the venter is thin, averaging some 4 μ in thickness. Its epicuticle is indistinguishable from that of other parts of the body.

A. The Procuticle

There is no exocuticle. Less than half of the procuticle consists of mesocuticle which displays no signs of the chemical sub-zonation characteristic of the dorsal mesocuticle. It has no visible internal structure and chemically, is typical of this zone generally.

The endocuticle comprises rather more than half of the procuticle, being thicker than that of the dorsum. No lipoid zone is present. Both chemically and structurally it resembles the endocuticle of *P. adonidum*.

3. *The Pleural Cuticle*

What is here assumed to be the cuticle of the degenerate pleura covers two narrow regions, one on either side, which connect the ventral and dorsal cuticles. From both of these the pleural cuticle differs greatly in structure. In transverse sections, its surfaces are irregular (Fig. 5) and its general appearance suggests that it is in a contracted state; it is possible that in the living insect it may be much more extended and correspondingly thinner.

It is frequently thick, exceeding 30 μ in a few places. The epicuticle is typical. The procuticle consists wholly of endocuticle which, when stained, shows no structure under bright-field conditions. When viewed under phase-contrast conditions, whether stained or not, numerous, fine, approximately-transverse dark lines can be seen. These appear to be artifacts produced by the contraction assumed to have occurred. The change from pleural to dorsal, or pleural to ventral, cuticles is sharp; there is no gradation of one region into the other.

Tests reveal the presence of the normal chitin-protein complex; there are no unusual components.

4. *The Cuticle of the Intersegmental Membranes*

Functional intersegmental membranes are found uniting the ventral abdominal segments only, so that cuticle of this kind is restricted to the ventral surface.

It is very thin and consists of the typical epicuticle overlying a procuticle rarely exceeding 2 μ in thickness and often being less. Its highly plicate condition in many places shows that it permits of considerable movement of the ventral region. The procuticle, which is composed wholly of endocuticle, has no visible internal structure, and no peculiar chemical properties. There appear to be no pore canals in it.

III. The Cuticle of E. acaciae (Maskell)

The cuticle of *E. acaciae* differs in details, only, from that of its congener. The species is somewhat smaller than is *Epicoccus* sp. but relative to the size of the insect its cuticle is still massive.

The epicuticles of both forms are indistinguishable even to the extent that dorsally the paraffin layer of each is covered by a sub-microscopic surface secretion of similar properties.

What has been said of the cuticles of the venter, the pleura, and the intersegmental membranes of *Epicoccus* sp., applies equally to those of the same regions of *E. acaciae*, such differences as do occur being confined to the dorsal procuticle.

The endocuticle of the latter has undergone still further reduction and forms irregular cappings to terminations of the cuticular ingrowths. It does not occur elsewhere. The procuticle thus consists almost entirely of mesocuticle which structurally resembles that of *Epicoccus* sp.; its distinguishing characters are chemical. Millon's reagent colors it uniformly cherry-red—a much deeper shade than is produced by the reagent in insect cuticle generally. The xanthoproteic test stains it uniformly deep orange, the iodine technique purple-black, ninhydrin violet-pink, and Mallory's PTAH deep purple. Sevki's technique displays the pore canals as deep violet, filamentous tubes of approximately constant diameter throughout their length and demonstrates their continuity between the hypodermis and the outer procuticular surface. After twelve hours' extraction at 60° C. with 10% hydrochloric acid, Millon's reagent stains the whole procuticle pink, and the xanthoproteic test colors it yellow. It fails to give visible responses with the other stains and reagents.

These results would seem to indicate that extra protein is present in the mesocuticle, that it is uniformly distributed (in contrast to its aggregated condition in *Epicoccus* sp.), and that it is differentiated into acid-extractable and acid-resistant fractions.

A lipoid zone, whose extent and reactions are identical with those of the corresponding zone of *Epicoccus* sp., is present. It differs in being brown pigmented with melanin or a melanin-like product, and is hence easily recognizable even in unstained sections.

IV. The Wax Glands and Their Secretion

(a) *P. adonidum*

Wax glands are conspicuous in most sections of *P. adonidum*, frequently as many as four or five, transected in various places, being visible in the one section. They resemble those of *P. martimus* as described by Pollister (1937) and it is probable that a general uniformity of glandular structure prevails throughout the family. Fundamentally, each consists of a multicellular secretory sheath enclosing a large, sub-spherical, central reservoir which communicates with the surface by means of one or more ducts (Fig. 1A).

When sections of unfixed material are treated with Sudan black B, the surface wax, the contents of reservoirs and ducts, and limited parts of the cuticle surrounding the ducts, are blackened. The local cuticular impregnation appears to be brought about by diffusion of some of the wax through the duct walls. The Nile blue sulfate technique stains the surface wax, that filling the ducts, and the impregnated cuticle deep blue, but it colors the reservoir contents red. Fat solvents readily dissolve the surface wax and that of the ducts but have no apparent effect on the impregnated cuticle or the reservoir material. The latter is highly resistant to such solvents; prolonged extraction with methanol-chloroform, pyridine, ether, or boiling cyclohexane removes part only of its lipoid. This explains why even in paraffin sections cut at 1 μ the contents of the reservoirs are retained apparently unaltered by the treatment they have undergone in the course of their preparation.

These results suggest that the material in the reservoir is "protowax" consisting of neutral lipoid (indicated by the Nile blue sulfate technique) so bound to other substances as to render its extraction extremely difficult. Alternatively the protowax may be secreted as an emulsoid whose finely divided lipoid micelles are dispersed in an inert medium. By some means at present unknown, the lipoid is freed and passed along the ducts. On its way to the surface the neutral lipoid becomes acidic, in which form it is deposited as wax on the surface. This interpretation of the course of events is purely speculative. An important obstacle to its acceptance is the failure of any of the wide range of tests applied, to demonstrate the presence in the reservoir of anything except lipoid.

There is some evidence suggesting that the outer surface of the wax layer may be covered by a sub-microscopic layer of protective material.

(a) If insects are killed with cyanide and then immersed in a solution of Sudan black B, the surface wax is stained only where it has been damaged during manipulation. If they are first lightly brushed, the areas so treated stain rapidly.

(b) If the insects have a prior immersion for fifteen minutes in 10% hydrochloric acid at 35° C. before being put in Sudan black B, staining of the wax is rapid and complete. Moreover, from insects so pre-treated, cold cyclohexane dissolves the wax rapidly whereas it acts much more slowly on untreated insects.

(c) If the insects are dropped into water at 70° C., the wax oozes away forming a surface film on the water. If such insects are then embedded in paraffin and sectioned, the epicuticle, when viewed under phase-contrast conditions, has an outer surface which cannot be sharply focussed. If the same sections are then treated with warm 10% hydrochloric acid for ten minutes, and re-examined, the epicuticle has a well-defined outer boundary.

Should an outer layer be present, the wax and its protective layer would afford an interesting analogy to the two outer layers of the four-layered type of epicuticle. They would occupy the same anatomical position relative to the two inner layers, and apparently perform the same function of limiting water loss as do the wax and cement layers. They differ in that the wax at least is a glandular product, they are not secreted until after moulting, and finally, some of the wax is used by many species as a covering for egg masses.

(b) *Epicoccus*

In *Epicoccus* most of the wax glands have atrophied. Their orifices are still open, but either their ducts are internally sealed off by the growth of procuticle across them, or their secretory cells are small and produce little wax. Glands of the latter kind are confined to the dorsum where they open in small groups in deep infolds of the cuticle adjoining muscle insertions. They secrete little more wax than is needed to plug the ducts and keep them filled. Their orifices are marked by small white spots at the surface (Fig. 4).

Typical glands are confined to the lateral extremities of the lobes in contact with the bark. The gland contents and the cementing wax respond similarly to stains and tests as do those of *P. adonidum*. The principal differences between the waxes of the two species are that that of *Epicoccus* has a higher melting point, and dissolves easily and quickly in all fat solvents. It would appear that the wax having ceased to function as an agent for reducing desiccation, no protective layer covers it.

V. Discussion

The results of this investigation demonstrate that notwithstanding the fundamental similarity of cuticular structure which prevails throughout the Pseudococcidae, great morphological differences distinguish the two genera studied.

A. The Epicuticle

The epicuticles of all three species consist of the essential cuticulin and paraffin layers. The paraffin layer of *Epicoccus* is overlain by a sub-microscopic covering of apparently lipoidal material which probably corresponds to the wax layer of more complex epicuticles, as a cement layer in this anatomical position would be abnormal. There is no indication of the presence of any such layer in *Pseudococcus* in which it is replaced by a thick layer of wax secreted by hypodermal glands; this surface wax itself appears to possess an extremely tenuous protective covering.

The effectiveness of either of these systems as a means for restricting water loss is probably slight. Much of the surface wax of *Pseudococcus*, for example, is disposed in long filaments which would have little value in this regard, more especially for a species which inhabits a humid micro-environment. Dead insects of either genus, after removal of the surface layer, do not lose water to a dry atmosphere at a significantly greater rate than do dead intact ones.

B. The Procuticle

The procuticles of the two genera differ as greatly as do the environments inhabited by each. The thin procuticle of *Pseudococcus*, consisting almost solely of endocuticle, displays little specialization. That of *Epicoccus*, on the contrary, is highly specialized both structurally and chemically.

In absolute thickness it is comparable with those of large sclerotized forms such as *Periplaneta*, while relative to the individual's size, there are probably few other insects which can match it. It consists almost wholly of mesocuticle.

Among insects whose cuticles have been described, that of *Epicoccus* is unique in the high proportion of protein in the procuticle, aggregated in a well-defined zone.

Its possession of a deep-seated lipoid zone in the procuticle is, so far as is known, shared only by the desert locust, *Schistocerca gregaria.*

All this specialization betokens a long period of evolution under conditions adverse to insect life generally, and the acquisition of a massive cuticle, together with the loss of mobility by the adult female, are presumably closely linked with this. Incapacity for locomotion may be disadvantageous; but failure to cope with a hostile environment spells extinction.

The collected material came from a single half-dead plant, not over ten feet in height. Fully-exposed as the insects were, they not infrequently had to contend with direct sun temperatures of 60° C., or even more, accompanied by relative humidities often below 10%, in a situation commonly swept by parching winds. The brood chambers of mature females contained eggs, and first and second nymphal instars. These immature forms were found nowhere else. Apart, therefore, from ensuring survival of the mother, the thick leathery cuticle functions equally well in protecting those stages in the life cycle most vulnerable to the environmental conditions, thereby making it possible for the species to maintain itself in a region relatively-poor in the higher forms of insect life.

I wish to thank Miss Helen M. Brookes of this department for supplying the material used and for allowing publication of the photograph reproduced in Figure 4.

My thanks are also due to Mr. Keith P. Phillips (in charge of Photographic Department) who is responsible for all the photography.

SUMMARY

1. The cuticular structure of three female pseudococcids, *Pseudococcus adonidum* L., *Epicoccus* sp., and *E. acaciae* (Maskell), has been investigated.

2. The cuticle of *P. adonidum* consists of a two-layered epicuticle, overlying a thin procuticle, almost all of which is endocuticle.

3. The cuticle of *Epicoccus* sp., is highly specialized. Its epicuticle closely resembles that of *P. adonidum.* The dorsal cuticle is relatively thick, and is much modified chemically. Most of it consists of mesocuticle in which Millon's reagent delimits three well-defined zones which differ greatly in their reactions to stains and histochemical reagents. The endocuticle is much reduced. A thin layer of procuticle between the hypodermis and the endocuticle is impregnated with lipoid to from a "lipoid zone."

4. The cuticle of *E. acaciae* is thick. It differs from that of *Epicoccus* sp. principally in that there is no chemical zonation of the procuticle, and the lipoid zone is melanin-pigmented.

5. Wax glands are numerous in the cuticle of *P. adonidum.* The contents of their reservoirs ("protowax") differ chemically from the surface wax. In *Epicoccus*, many of the glands have atrophied; typical glands are confined to lateral parts of the cuticle in contact with the host plant, and these secrete large quantities of wax which fixes the insect permanently in position.

6. The specialized cuticle of *Epicoccus* appears to have evolved over a long period, during which the insects have been exposed to adverse environmental conditions.

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PHENYLTHIOUREA TREATMENT AND BINDING OF RADIOACTIVE IODINE IN THE TADPOLE

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In a study of the distribution of I^{131} after administration to tadpoles, Dent and Hunt (1952) demonstrated not only the expected concentration of this substance in the thyroid gland but also significant accumulations in several other regions. Notable among these were the thymus, the horny teeth, the melanophores of the skin, and the pigmented layer of the retina. The same pattern of distribution was observed in tadpoles of *Hyla, Rana,* and *Bufo* in various stages of development and was not altered by thyroidectomy. It was suggested that, since tyrosine is a precursor of melanin, the localization of I^{131} in pigmented tissues may be attributable to the binding of I^{131} to tyrosine that must be present in those tissues. The hypothesis was also advanced that perhaps the same enzymes that bring about oxidation of tyrosine to melanin are able to facilitate the union of iodine and tyrosine. Gennaro and Clements (1956a, 1956b), studying the binding of I^{131} in the skin of the adult frog, provided evidence to support these views. Our experiments were undertaken as a further investigation of the association of iodine with pigmented tissues and as a further test of the hypothesis cited.

It is known that administration of certain derivatives of thiourea results in an inhibition of melanin formation. This has been demonstrated for mammals (Richter and Clisby, 1941; Dieke, 1947), fishes (Frieders, 1954), and several different amphibians (Lynn and de Marie, 1946; Lynn, 1948; Blackstad, 1949; Millott and Lynn, 1954). In amphibians the results are most striking when these substances are given to embryos or young larvae before any melanophores have appeared. Such individuals do not develop black pigment so long as the treatment is continued. Cessation of treatment is followed by rapid melanogenesis. Since it has been demonstrated (Bernheim and Bernheim, 1942; Paschkis, Cantarow, Hart and Rakoff, 1944; Dubois and Erway, 1946) that thiourea derivatives inhibit tyrosinase activity *in vitro,* it is assumed that their role in preventing melanin formation in frog embryos is the inhibition of tryosinase activity. In our experiments, larvae of several different ages were treated with one of these tyrosinase inhibitors, phenylthiourea, to obtain unpigmented tadpoles or tadpoles with reduced pigmentation. The pattern of iodine uptake in these animals was compared with that in untreated controls at various times after the beginning of treatment.

MATERIALS AND METHODS

The animals used for this experiment were tadpoles of *Hyla versicolor versicolor* LeConte hatched from eggs collected in a small temporary pool near Oak Ridge,

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Tennessee. A few hours after hatching, the larvae were distributed in groups of fifteen in finger bowls, each containing 200 ml. of spring water. On the first, fourth, and fifteenth days after hatching, experimental series were established in this way: Larvae were transferred to six finger bowls, fifteen larvae to each bowl; two contained 200 ml. of 0.01% phenylthiourea in spring water, two contained 200 ml. of 0.005% phenylthiourea in spring water, and two contained spring water alone (controls). The tadpoles, kept at laboratory temperatures (21°–23° C.), were fed crumbled pellets of Purina rat chow and, occasionally, a strained beef-and-liver soup prepared for infants. The culture fluids were changed daily and there was no mortality. At four-day intervals three animals from each experimental and control group were selected at random and examined under the binocular microscope. Records were kept of the gross changes in pigmentation and of the developmental stage reached. The system of staging devised by Taylor and Kollros (1946) for *Rana pipiens* was used and adapted with minor variations for *Hyla versicolor*.

At three ages (4, 24, and 29 days after hatching), animals were removed from the experimental and control series and used in the preparation of autoradiograms.[4] The procedure was as follows: Five larvae from each bowl were put in 50 ml. of a solution of one part per million of stable sodium iodide [5] and enough radioiodine to give an activity of one μc./ml. at the beginning of the immersion period. For each experimental group, one set of five animals was put in a radioiodine solution made up in spring water and another was put in a radioiodine solution made up in the same phenylthiourea solution in which the animals had been raised. After 24 hours in the radioiodine solution, the larvae were passed through two baths of spring water and left in a third bath of spring water, or the appropriate phenylthiourea solution, for another 24 hours. All were then fixed in a 1:1 mixture of Bouin's fluid and Cellosolve. After 8 hours' fixation, they were dehydrated in Cellosolve, embedded in paraffin, and sectioned at 10 micra. The mounted and dried sections were passed through two changes of xylol, transferred to absolute alcohol, and then dipped in a 1.0% solution of collodion in ether-alcohol, and dried. The slides were attached by stationer's binder clips against the emulsion of Eastman medium contrast lantern slide plates in the darkroom and left for 8 days. Finally, the lantern slide plates were developed and the sections themselves were stained with Harris' haematoxylin and Ponceau de xylidine-orange II (Gray, 1952).

RESULTS

1. *Gross effects of treatment with phenylthiourea*

During ovogenesis (Kemp, 1953) melanin granules are laid down in the cortical region of most anuran ova. Superficially, the pigmented area extends from the animal pole to the presumptive germ ring. These granules are retained within

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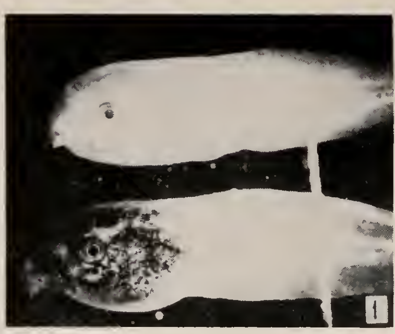
[4] Joftes and Warren (1956) have recommended the substitution of the term "radioautogram" for "autoradiogram." It is felt that since the semantic basis offered by Joftes and Warren for the use of the former term does not appear to be much stronger than the etymological basis presented by Boyd (1955) for the use of the latter and since the latter term has become well established, its use should be continued.

[5] There is some danger of introducing errors by use of carrier-free isotopes because of their tendency to adhere to glassware. The stable NaI was added with the view of eliminating that effect.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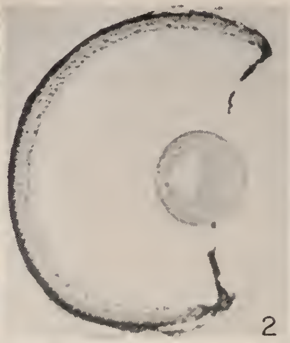
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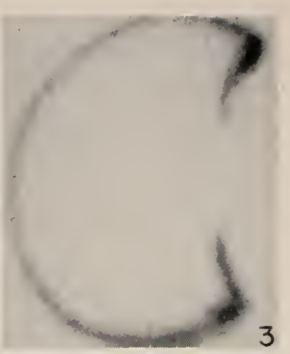
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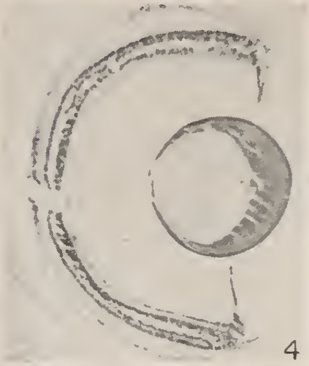
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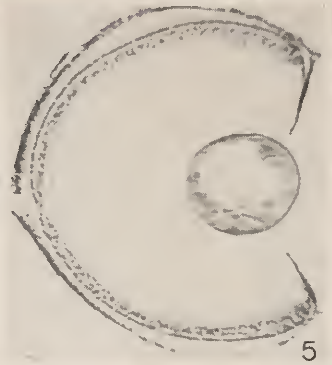
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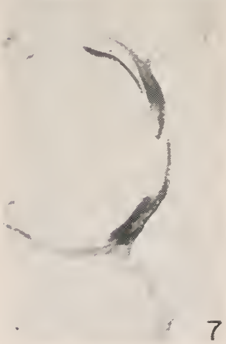
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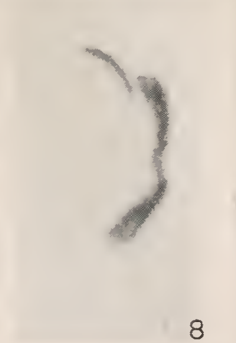
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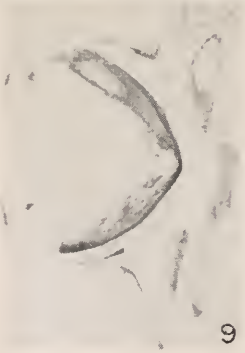
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All sections shown are from 26-day-old larvae of *Hyla versicolor* that had been immersed in a solution of one μc of I^{131}/ml. for 24 hours and fixed 24 hours after removal from the solution.

FIGURE 1. Photograph of living tadpoles 12 days old. Upper animal raised in 0.01% phenylthiourea; lower animal raised in spring water.
FIGURE 2. Section through the eye of a control tadpole.
FIGURE 3. Autoradiogram prepared from the section shown in Figure 2.
FIGURE 4. Section through the eye of a tadpole kept in 0.01% phenylthiourea continuously.
FIGURE 5. Section through the eye of a tadpole kept in 0.01% phenylthiourea for 24 days, then in spring water for two days.
FIGURE 6. Autoradiogram of the section shown in Figure 5.
FIGURE 7. Section through the mouth of a control tadpole showing the horny teeth.
FIGURE 8. Autoradiogram of the section shown in Figure 7.

the presumptive ectoderm and mesoderm but disappear as melanophores begin to differentiate and form melanin.

As was anticipated on the basis of previous experiments with other amphibians, the larvae that had been put in phenylthiourea solutions within a few hours after hatching stopped forming melanin and after only 24 hours were noticeably paler than controls. After 48 hours, and at all later stages, the experimental animals were completely unpigmented, the embryonic pigmentation having disappeared (Fig. 1). Larvae in which treatment was delayed until four days after hatching had already developed much melanin in both skin and eyes and exhibited no difference from the controls for at least three days. After this they gradually paled, however, and by the tenth day of treatment their skin was without pigment. Pigmentation of the eyes was lost much more slowly and even when the experiment was terminated (51 days after hatching), the eyes of these animals still showed some pigment, though far less than those of controls. Larvae that were first put into phenylthiourea solutions 15 days after hatching showed no blanching of the skin until near the end of the experiment, and the pigmentation of the eyes never became grossly different from that of controls. Examination of the living animals under the binocular microscope and later study of the sectioned material revealed no significant pigmentary difference between animals treated with 0.01% phenylthiourea and those treated with 0.005%. It appears that both concentrations are fully effective in inhibiting melanin formation.

Since phenylthiourea is one of the well-known thyroid-inhibiting drugs, the experimental animals not only differed from the controls in degree of pigmentation but also in their failure to exhibit definitive metamorphic changes. No significant differences in development were noted for the first 20 days of the experiment. At this time both treated and untreated tadpoles were in late limb bud stages (Stages IV and V). Later, however, the experimental animals showed definite inhibition in development. By 24 days the controls were in Stages VI and VII whereas the tadpoles in phenylthiourea solutions remained at Stages IV and V. The controls continued to differentiate steadily, most specimens reaching Stage IX by the 28th day, Stage XV by the 40th day, and late metamorphic stages (Stages XVIII to XXV) by the 48th day. As is usual with anuran larvae, there was a considerable variation in developmental rate among the controls, a few specimens being retarded and others exceptionally advanced. Thus, although forelimb emergence occurred in one control 45 days after hatching, and had occurred in more than half the surviving controls by 51 days, at this latter time there was still one animal at Stage VI and several at Stage XIII. Among the tadpoles placed in 0.01% phenylthiourea, either immediately after hatching or 4 or 15 days later, none advanced beyond Stage VI and most remained at Stage IV or Stage V. Tadpoles raised in 0.005% phenylthiourea advanced somewhat beyond those in the higher concentration; most of those kept to the end of the experiment reached Stage VI or Stage VII and a few specimens differentiated to Stage VIII. There is thus some indication that the lower concentration is not fully effective in inhibiting thyroid activity.

FIGURE 9. Section through the mouth of a tadpole treated continuously with 0.01% phenylthiourea.

FIGURE 10. Autoradiogram of the section shown in Figure 9.

As noted previously, the larvae used for the preparation of autoradiograms were put in a radioiodine solution for 24 hours, and this was followed by another 24-hour period without radioiodine, which allowed for elimination of excess iodine before fixation of the tadpoles. Autoradiograms were made from two sets of phenylthiourea-treated animals, one in which the phenylthiourea treatment was continued until fixation and one in which the radioiodine solution and the subsequent 24-hour bath were without phenylthiourea. The latter thus had a 48-hour period of recovery from phenylthiourea treatment immediately preceding fixation. It has been demonstrated that in amphibian embryos melanin reappears very rapidly after phenylthiourea treatment has stopped (Millott and Lynn, 1954). In the present experiments the animals put in radioiodine solutions containing no phenylthiourea showed a well-defined darkening of the eyes within 12 hours; and at 24 hours, when they were removed from the radioiodine solutions, a scattering of pigmented melanophores was also visible in the skin. Larvae kept in phenylthiourea solutions throughout, of course, showed no such pigmentary change.

2. *Effects of phenylthiourea treatment on radioiodine binding in the pigmented epithelium of the retina*

The first group tested for radioiodine binding consisted of animals on which the phenylthiourea treatment started on the day of hatching and continued for four days only. The second and third groups tested included animals on which treatment was begun on the day of hatching and some in which treatment was started later (4 and 15 days after hatching). Since the results were similar in all three groups, detailed consideration will be given for only one, the second experimental group, which was given radioiodine on the 24th day after hatching and contained animals under treatment for 24, 20, and 9 days.

Photomicrographs and corresponding autoradiograms of the eye region in typical animals of this group are shown in Figures 2–6. Figures 2 and 4 illustrate the conditions found in control and experimental animals of the series in which phenylthiourea treatment was started at hatching. The pigmented epithelium of the retina is quite dark in the controls but is entirely without melanin in the animals given continuous phenylthiourea treatment. The autoradiogram prepared from the control (Fig. 3) shows that there was a marked concentration of radioiodine in the pigment epithelium. On the other hand, an autoradiogram prepared from the eye shown in Figure 4 was entirely blank and is not shown. It has been pointed out that some melanin formed in the animals allowed a recovery period from treatment with phenylthiourea. Plates prepared from these specimens show faint but definite autoradiograms, indicating that some binding of radioiodine occurred both in the skin and in the retina (Figs. 5, 6). On the basis of these results it might be concluded that the binding of radioiodine depends on the presence of melanin and varies directly in amount with the amount of melanin present. However, study of the larvae in which phenylthiourea treatment was initiated at later ages reveals that this is not the case. It will be remembered that pigment was lost from the eyes only very slowly in the series started at 4 days and not at all, so far as could be seen externally, in the series started at 15 days. Thus these latter tadpoles, even though under continuous treatment with phenylthiourea, still had much melanin in the pigment epithelium. Nevertheless the autoradiograms prepared from this series exhibited the same variations as those previously described. Control animals showed high radioiodine level, animals treated continuously with phenylthiourea showed no radioiodine binding, those treated with phenylthiourea and then allowed two days' recovery showed a very low radioiodine level. Yet the degree of pigmentation of the members of all groups was about the same. It is therefore clear that the binding of radioiodine does not depend on the amount of formed melanin present.

3. *Effects of phenylthiourea treatment on radioiodine binding by the horny teeth*

In all larvae given radioiodine a significant localization was found in the horny teeth. The 4-day series (fixed at 6 days after hatching) shows relatively little cornification of the teeth. Nevertheless the autoradiograms indicate that radioiodine was bound in these structures, not only in the controls but also, and apparently to the same extent, in the phenylthiourea-treated animals. The 24- and 39-day series both exhibit extensive cornification of the teeth, and in these there is an indication that phenylthiourea did lessen the binding of radioiodine without, however, completely halting it. Photomicrographs and corresponding autoradiograms of the teeth in control and experimental animals of the 24-day series will serve to illustrate this inhibitory effect (Figs. 7–10). It was found that whereas the autoradiograms of control tadpoles (Fig. 8) and those of treated animals allowed a 48-hour recovery period show equally dense spots, representing the teeth, autoradiograms of larvae subjected to continuous treatment with phenylthiourea show definite but much less intense darkening (Fig. 10). This result was consistent in all specimens of the older series.

4. *Effects of phenylthiourea treatment on the thyroid and thymus*

Although these experiments were not primarily concerned with the thyroid gland, some observations on the thyroid response are of interest. In most of the control larvae of the 4-day series the thyroid proved to be at a stage of early follicle formation. Only one or two follicles were present in each thyroid, the rest of the gland consisting of irregular cords of cells. The formed follicles had a cuboidal epithelium that still retained some of the pigmentation characteristic of the anuran thyroid rudiment. The lumina were very small and contained no stained colloid. Two of the five control specimens had no organized follicles at all, the entire thyroid being composed of clumps and cords of epithelial cells. In the experimental animals of this series, there were some with glands lacking organized follicles; these showed no histological differences from the two controls just mentioned. On the other hand, the phenylthiourea-treated larvae in which follicles had appeared showed a sharp contrast to controls in that the follicles were larger, had a flattened epithelium, and contained a relatively large amount of homogeneous basophilic colloid. The autoradiograms of the control thyroids and those allowed 48 hours' recovery from phenylthiourea showed evidence of binding of radioiodine, whereas the animals treated continuously with phenylthiourea did not. It is noteworthy that the thyroids of all 5 of the controls and of all 5 of the recovery series produced clear autoradiograms but in some of them no organized follicles were yet present. The effect of phenylthiourea treatment on thyroid physiology is thus detectable histologically as soon as follicles are formed and physiologically even before follicles are formed.

The thyroids of controls fixed at 26 days after hatching showed a quite uniform appearance. The follicular epithelium was cuboidal and the colloid acidophilic, usually with some chromophobe droplets. Experimental animals differed consistently in having many more chromophobe droplets and slightly higher epithelium. The different lengths of treatment with phenylthiourea resulted in no histologically observable differences. There was also no significant difference in the appearance of the thyroids of animals treated continuously and those allowed two days' recovery before fixation. Autoradiograms prepared from the sections of this series showed a high level of radioiodine in the control thyroids, a very low level in the thyroids of larvae treated continuously with phenylthiourea, and a high level, apparently as high as that of controls, in those of larvae removed from phenylthiourea at the time of their exposure to radioiodine solution.

The controls of the series fixed at 41 days after hatching were all in metamorphic stages and, as would be expected, their thyroids gave indications of high activity. The epithelium tended to be columnar and chromophobe droplets were abundant. The experimental animals, on the other hand, had enlarged follicles with a markedly flattened epithelium and much homogeneous colloid. The autoradiograms of this series are similar to those for the 24-day series.

The concentration of radioiodine in the thymus gland observed by Dent and Hunt (1952) was confirmed in these experiments and proved to be affected by phenylthiourea treatment in exactly the same way as is radioiodine concentration in the thyroid. Autoradiograms made from control tadpoles of the 24-day series or from tadpoles allowed a recovery period show high concentration of radioactivity in the thymus; those made from tadpoles under continuous treatment with phenylthiourea show no radioactivity in this region.

DISCUSSION

In these experiments the administration of phenylthiourea to early larvae of *Hyla versicolor versicolor* resulted in the production of completely unpigmented tadpoles. Both concentrations tested (0.01 and 0.005%) proved equally effective and neither gave any indications of toxicity. The gradual blanching of the skin produced by treatment with phenylthiourea has sometimes been referred to as a depigmentation effect. It is probable, however, that the drug has no effect on any pigment already present when treatment is begun but acts entirely by preventing the formation of new pigment. The results of our experiments are in accord with this view for, as has been pointed out, the blanching of the skin (and of the eyes) occurred rapidly in larvae treated immediately after hatching, more slowly in those in which treatment was delayed until 4 days after hatching, and very slowly indeed in those in which treatment was begun 15 days after hatching. It must be assumed that in all these animals the phenylthiourea treatment effectively blocked melanogenesis and that the rate of "depigmentation" depended on the rate of loss of the melanin already present. In fact, it would appear that this rate of blanching after treatment with phenylthiourea should furnish an indication of the normal rate of metabolic turnover of melanin at various ages. Our results indicate that turnover is rapid at early ages but much slower in older animals. In fact, it seems likely that some of the formed melanin persists indefinitely after a certain age is reached.

These experiments also demonstrate that phenylthiourea affects the binding of radioiodine by the tapetum nigrum. Only the untreated animals show a significant concentration of I^{131} by this structure. Tadpoles given phenylthiourea and then removed from the solution exhibit an ability to bind iodine within the first 24 hours after cessation of treatment. In all experiments, however, the autoradiograms, though they do not give quantitative information, indicate clearly that I^{131} is not taken up by the pigmented epithelium of the eye in direct proportion to the amount of melanin present. Tadpoles for which phenylthiourea treatment is begun at 15 days after hatching retain much pigment in the eye yet show no tendency to bind I^{131}. This indicates that the binding of iodine in the pigmented epithelium of the eye (and doubtless in chromatophores as well) takes place only while melanin is actually being formed and is dependent on some enzymatic activity that is inhibited by phenylthiourea. Since this substance is known to inhibit tyrosinase activity *in vitro* and since tyrosine must be present where melanogenesis is going on, it is natural to suspect that tyrosinase is the enzyme involved. These views are supported by the findings of Gennaro and Clements, who extracted radioactive mono- and diiodotyrosine from discs of frog skin that had been incubated in Ringer solution containing I^{131} and from the skins of intact frogs injected with I^{131} (1956a). They also showed that pretreatment of the discs with thiourea decreased the degree of incorporation of I^{131} in the melanized areas (1956b). According to the concept outlined, tyrosinase would be active in pigment-forming tissues both in the oxidation of tyrosine to melanin and in the oxidation of iodide to iodine to permit the production of mono- and diiodotyrosine and possibly iodinated proteins. Inhibition of tyrosinase activity would thus be expected to result simultaneously in cessation of melanogenesis and failure to bind I^{131}. Whether these findings can be directly related to the goitrogenic effects of phenylthiourea is not certain. The mechanism by which iodination of tyrosine occurs in the thyroid is not well understood (Roche and Michel, 1955). There is no evidence of the presence of tyrosinase in the mammalian thyroid (Pitt-Rivers, 1950). Fawcett and Kirkwood (1954) have hypothecated a "tyrosine iodinase" as a catalyst for the process. There are many varieties of tyrosinase (Lerner and Fitzpatrick, 1950), however, and it may be that amphibian tyrosinase has a special property of oxidizing iodine or, on the other hand, that our experiments may offer the key to a better understanding of iodination of tyrosine in the thyroid itself.

The accumulation of iodine in the horny teeth of larval anurans was first reported by Dent and Hunt (1952). Association of iodine with similar hard structures is known to occur in a number of invertebrates. Noteworthy examples are the hypodermis of Drosophila larvae (Wheeler, 1950), the setae and pharyngeal teeth of polychaetes (Swan, 1950), and the exoskeleton of Daphnia (Gorbman, Clements and O'Brien, 1954). Gorbman (1955) has discussed this matter in some detail and notes that in all these cases the localization of radioiodine is in scleroprotein. The present experiments indicate that phenylthiourea treatment, if given over a sufficiently long period, has an inhibitory effect on the binding of radioiodine here although it does not completely prevent it.

The effects of derivatives of thiourea on the functioning of the thyroid gland have been widely studied in mammals and in several amphibians. The histological changes seen in the thyroids of the animals studied in our experiments are in agreement with those previously reported and need not be discussed. The autoradiographic analysis of the thyroids of the control and experimental animals also gave results that would be expected on the basis of what is known of the effects of this drug. Larvae under continuous treatment with phenylthiourea showed an extremely low ability to bind I^{131}. However, treated larvae recovered this ability very rapidly after treatment was discontinued. This rapid rate of recovery is in contrast with the slower rate observed in the pigmented regions and may be indicative of a difference in the mechanism of iodine binding.

The basis for the accumulation of radioiodine by the thymus gland is not known. It was first reported by Dent and Hunt (1952), and it is clearly demonstrated in our material. It is completely inhibited in animals under continuous treatment with phenylthiourea but cessation of treatment is followed by prompt recovery of the animal's ability to concentrate iodine. It appears, then, that the binding of iodine in the thymus is more closely related to that process as it occurs in the thyroid than as it occurs in the melanophore.

Earlier observations on various vertebrates (see citations in Dent and Hunt, 1954) have all been to the effect that the onset of iodine accumulation by the thyroid does not occur until discrete follicles make their appearance. It is of some interest, then, that in the animals studied here iodine concentration began while the cells of the thyroid rudiment were still arranged in cords at four days after hatching. Moreover, such animals, when given continuous phenylthiourea treatment, showed no ability to concentrate I^{131}. Thus the iodine-concentrating activity of thyroid tissue, and also the ability of phenylthiourea to inhibit this activity, are evidenced well before the appearance of follicles or colloid.

SUMMARY

1. Larvae of *Hyla versicolor* were immersed in solutions of phenylthiourea at 0, 4, and 15 days after hatching. At 4, 24, and 39 days after hatching, I^{131} was administered and contact autoradiograms were prepared from serially sectioned representative specimens.

2. The tadpoles treated with phenylthiourea from the time of hatching became completely unpigmented. The blanching of the second series was slower and never complete. The third series became very little lighter during the course of the investigation. This indicates that the metabolic turnover of melanin goes on at a decreasing rate as the larvae increase in age.

3. From the autoradiograms, evidence was obtained to confirm earlier findings of the binding of iodine in pigmented areas, to show that the binding is apparently not associated with formed melanin, and to support the view that the same enzyme or enzymes that catalyze melanogenesis can catalyze the binding of iodine (presumably with tyrosine).

4. The accumulation of iodine by the horny teeth was inhibited to some degree by phenylthiourea treatment.

5. Accumulation of radioiodine by the thymus gland was confirmed and was found to be completely inhibited by phenylthiourea treatment.

6. The thyroid rudiment acquires the facility for concentrating iodine even before follicle formation begins, and at that time it also responds to the inhibitory action of phenylthiourea.

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ENDOPARASITIC POLYCHAETOUS ANNELIDS OF THE FAMILY ARABELLIDAE WITH DESCRIPTIONS OF NEW SPECIES[1]

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Among the polychaetes, which for the most part are free-living, crawling, burrowing, and tube-dwelling, commensalism is rather common but internal parasitism is rare indeed. Among the relatively few cases that have been reported are some lumbrinerid-like polychaetes belonging to the superfamily Eunicea, which includes the families Eunicidae, Onuphidae, Lumbrineridae, Arabellidae, Lysaretidae, and Dorvilleidae (sometimes considered as subfamilies belonging to the family Eunicidae). Some of the parasitic euniceans invade other members of the same superfamily and may attain an enormous size in proportion to the host. All of the known lumbrinerid-like parasites belong to, or show affinities to, the family Arabellidae as defined by Hartman (1944).

In connection with a study of polychaete material from various sources, including that in the United States National Museum, some arabellids were found living as endoparasites in other polychaetes of the related family Onuphidae. Two new species are described herein, the types of which are deposited in the United States National Museum. Some small specimens of a third species, living parasitically in the onuphid, *Diopatra*, are thought to be the young stages of the arabellid, *Notocirrus spiniferus* (Moore). Since this interesting type of parasitism is not widely known and has not received the attention it no doubt deserves, the relatively few known cases of lumbrinerid-like species living in other polychaetes and echiuroids are reviewed and the chief characteristics of the Eunicea and Arabellidae to which the parasites belong are summarized.

Superfamily EUNICEA

All the members of the Eunicea are equipped with a complex series of strong, dark, chitinous or horny jaws. The pharynx is capable of protrusion and is provided with a pair of ventral plates, called mandibles, and a more dorsal bilaterally arranged series of plates, called maxillae. The prostomium is distinct, with or without eyes or appendages. Typically the first two segments are apodous. The parapodia are essentially uniramous, the upper lobe or notopodium represented at most by a few embedded notoacicula and a rudimentary papilla-like lobe. The members of the Eunicea vary from those of minute size with a moderate number of segments to some very large ones with very numerous segments, some of which are among the largest of the polychaetes. They are essentially free-living, predaceous, and carnivorous. They secrete abundant mucus, which may aid in burrowing or forming temporary or more or less permanent tubes.

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Family ARABELLIDAE Hartman

The arabellids show a superficial resemblance to the members of the Lumbrineridae, differing from them in setal and pharyngeal characters. They have the body elongate, cylindrical, of nearly uniform width, and tapering slightly anteriorly and posteriorly. The prostomium is reduced to a simple, conical or flattened spatulate lobe, without appendages, with or without eyespots on the posterior margin. The first two segments are distinct, apodous, and without appendages. The parapodia are essentially uniramous; the notopodia sometimes represented by a minute papillar lobe (sometimes referred to as a reduced dorsal cirrus) with embedded notoacicula; the neuropodia are unequally bilobed, with shorter rounded and longer digitiform postsetal lobes. The neurosetae are simple (not compound), limbate, and taper to fine tips; in addition they sometimes have projecting thick acicular setae or acicula (without hooded hooks as in the Lumbrineridae). The parapodia have no dorsal or ventral cirri or branchiae. The eversible proboscis is equipped with strong, chitinous, black jaw pieces: usually with a pair of ventral, flat plates, the mandibles; with 4 or 5 pairs (may be fewer in parasitic forms) of more dorsal maxillae arranged in parallel rows, with a pair of long filiform carriers to which a shorter median unpaired piece is attached on the ventral side. The arabellids are essentially a burrowing, predaceous, carnivorous group. They burrow readily but rather slowly in sand or mud. They secrete a good deal of mucus, which probably serves to lubricate the burrow.

Table I

Parasite	Host and Distribution	References
Notocirrus sp. (young)	Eunicidae: *Marphysa sanguinea* (Montagu). Mediterranean	Koch, 1847. Ehlers, 1868, p. 364. See below
Notocirrus ?spiniferus (Moore) (young)	Onuphidae: *Diopatra cuprea* (Bosc). Woods Hole region, Massachusetts	Allen, 1952. See below
Haematocleptes terebellidis Wirén	Terebellidae: *Terebellides stroemii* Sars. Off Sweden, 130 meters	Wirén, 1886
Labrorostratus parasiticus Saint-Joseph	Syllidae: *Odontosyllis ctenostoma* Claparède, *Syllis prolifera* Krohn, *Eusyllis monilicornis* Malmgren, *Pionosyllis lamelligera* Saint-Joseph, *Grubea clavata* (Claparède)	Saint-Joseph, 1888. Caullery and Mesnil, 1916. Fauvel, 1923, p. 440
Oligognathus bonelliae Spengel	Echiuroidea: *Bonellia viridis* Rolando. Mediterranean	Spengel, 1882. Fauvel, 1923, p. 442
Oligognathus parasiticus Cerruti	Spionidae: *Spio mecznikowianus* Claparède. Mediterranean	Cerruti, 1909. Fauvel, 1923, p. 442
Drilonereis parasiticus (Caullery)	Terebellidae: Genus and species? Near Timor, Dutch East Indies, 73 meters	Caullery, 1914. See below
Drilonereis forcipes (Hartman)	Eunicidae: *Eunice* sp., possibly *E. antennata* Savigny. San Benito Island, Lower California, 66–81 fathoms	Hartman, 1944. See below
Drilonereis benedicti n. sp.	Onuphidae: *Onuphis magna* (Webster). Tampa Bay, Florida, 12 fms.	See below
Drilonereis caulleryi n. sp.	Onuphidae: *Onuphis (Nothria) conchylega* Sars. Off Massachusetts to off Virginia, 101–317 fms.	See below

Some of the arabellids are parasitic in other polychaetes (eunicids, onuphids, syllids, terebellids) and in echiuroids (*Bonellia*), living inside the body cavity or vascular body wall or even in the vascular system of the host, at least during their early developmental stages. The eight previously reported parasitic arabellids, along with the two species described herein, are summarized in Table I and in the illustrated Key to the genera and species.

Illustrated Key to the Genera and Species of Parasitic Arabellidae

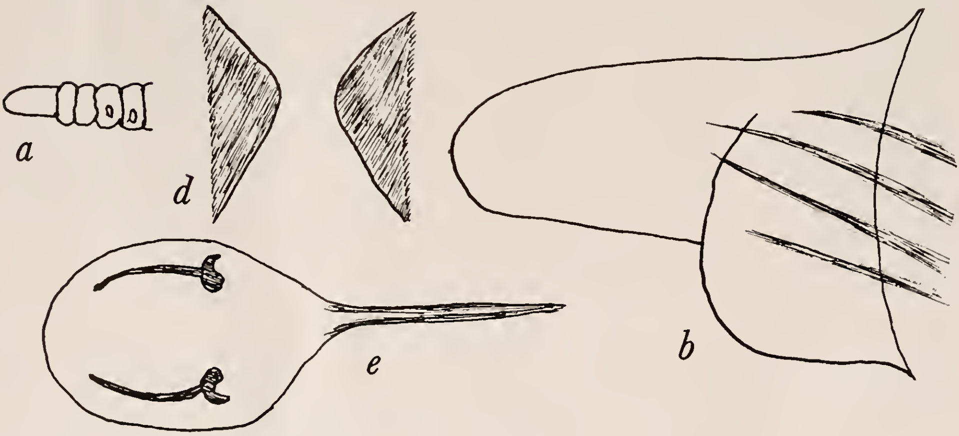
[Figures are copied from the original descriptions. a, anterior end; b, parapodium; c, setae; d, mandibles; e, maxillae and maxillary carriers; f, maxillae]

A¹. Numerous specimens in single host.

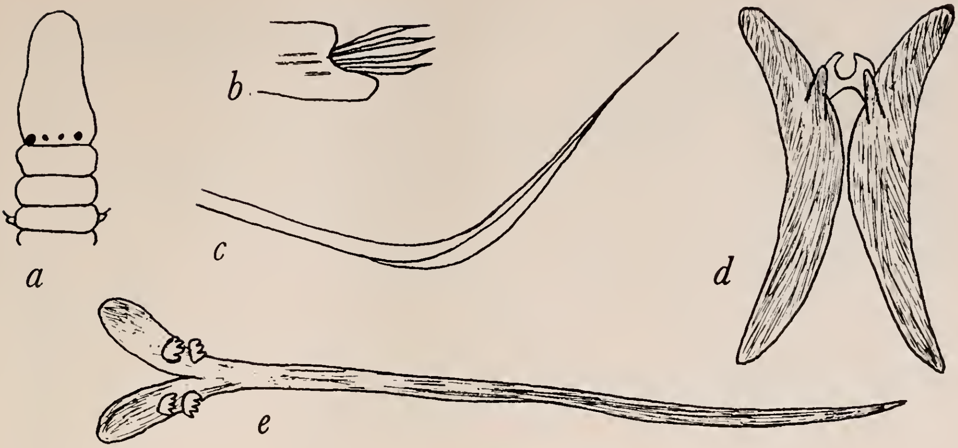
- B¹. Parasitic in body cavity of *Marphysa sanguinea* . . . *Notocirrus* sp. (young). (See below under *N. spiniferus.*)
- B². Parasitic in body cavity and vascular body wall of *Diopatra cuprea* *Notocirrus ?spiniferus* (young). (See Figs. 4, g and 5.)

A². One parasite per host.

- C¹. One to three pairs of rudimentary maxillae, with single elongate rodlike maxillary carrier. Mandibles present.
  - D¹. Maxillae a single pair of curved rods, each curved tooth at base. Setae and acicula not projecting from lobe. Mandibles paired, triangular. Prostomium without eyes. (Up to 25 mm. long, about 200 segments. Colorless.) Parasitic in peri-intestinal blood sinus of *Terebellides stroemii.* . . . Genus *Haematocleptes* Wirén *H. terebellidis* Wirén

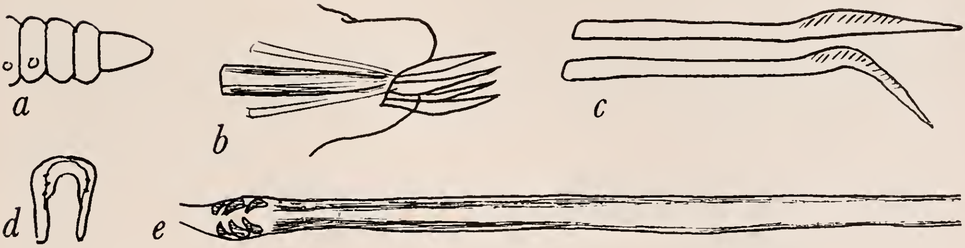


  - D². Two or three pairs of maxillae. Setae projecting from parapodial lobe.
    - E¹. Maxillae two pairs of very small denticled pieces. Mandibles wing-shaped, each with a spine. Setae all of one kind, limbate, smooth, tapering to long flexible tips. Prostomium with four eyes in transverse row. (Up to 70 segments.) Parasitic in body cavity of syllids, *Odontosyllis ctenostoma, Syllis prolifera, Eusyllis monilicornis, Pionosyllis lamelligera, Grubea clavata.* Also found free among calcareous algae, *Lithothamnion.* Genus *Labrorostratus* Saint-Joseph *L. parasiticus* Saint-Joseph

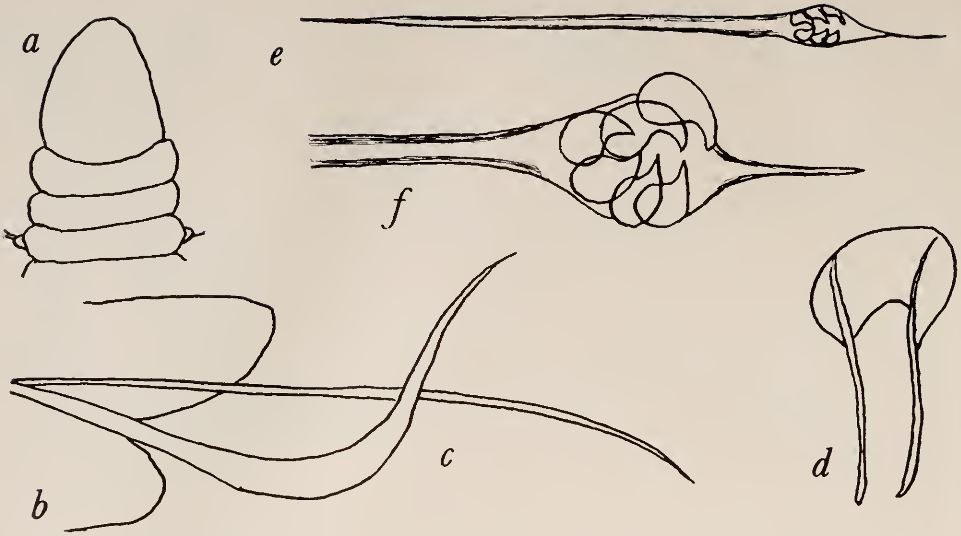


E² Maxillae three pairs recurved unidentate hooks. Mandibles U-shaped, with two wing-like pieces or rods united by transverse band. . . . Genus *Oligognathus* Spengel

F¹. Setae of one kind, simple, arched, limbate, striated. Prostomium with four eyes. (Up to 100 mm. long, more than 200 segments. Bright orange yellow.) Parasitic in body cavity of echiuroid, *Bonellia viridis*.
O. bonelliae Spengel



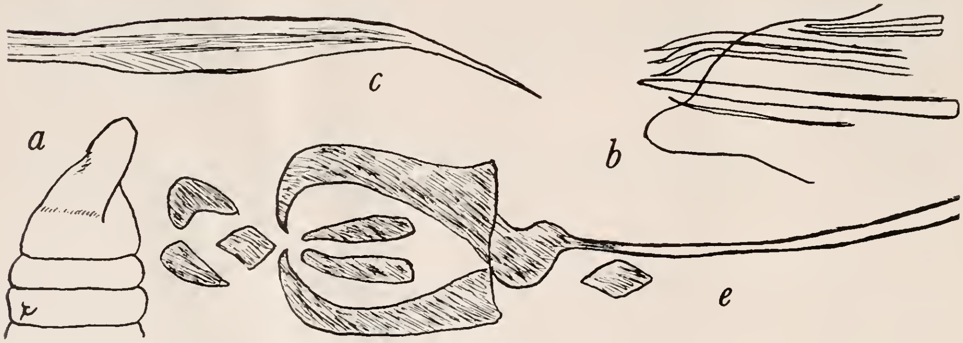
F². Setae of two kinds: capillary, flexible and stouter, wide, tapering to fine tips. Prostomium without eyes. (Up to 8 mm. long. 50 segments. Colorless, transparent.) Parasitic in body cavity of spionid, *Spio mecznikowianus*.
O. parasiticus Cerruti



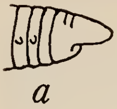
C^2. Four pairs of well developed maxillae, with pair of elongate rodlike maxillary carriers and shorter unpaired piece (basal or maxillae I large, strong, hooked, forceps-like; maxillae II rectangular plates, sometimes denticled; maxillae III and IV each a single strong thorn-like tooth). Mandibles absent. Parapodia with heavy acicula or acicular setae, the tips of which usually protrude. Prostomium without eyes.

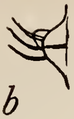
Genus *Drilonercis* Claparède (includes *Labidognathus* Caullery; see below).

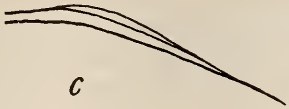
G^1. Parasitic in peri-intestinal blood sinus of terebellid (unidentified). With bilimbate setae and single stout acicular seta extending out of parapodial lobe. Maxillae II edentate (?, incompletely observed). (More than 100 segments). . . . *D. parasiticus* (Caullery)

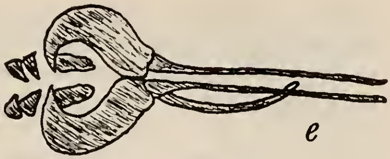


G^2. Parasitic in body cavity of branchial fragment of eunicid, *Eunice* sp., possibly *E. antennata*. With bilimbate setae and single stout yellow aciculum, the latter not extending out of parapodial lobe. Maxillae II flat plates, practically without teeth. (More than 140 segments, more than 30 mm. long.). . . . *D. forcipes* (Hartman)









G^3. Parasitic in body cavity of onuphids. Maxillae II rectangular plates, each with four distinct teeth.

H^1. Parasitic in branchial fragment of *Onuphis magna.* Without setae or acicular setae visible externally (even in a specimen of more than 1200 crowded segments and more than 240 mm. long). . . . *D. benedicti* n. sp. See Fig. 1, a-g.

H^2. Parasitic in anterior fragment of *Onuphis* (*Nothria*) *conchylega.* With limbate setae and single stout acicular seta extending from lobe (except in smaller specimens; up to 400 segments, 110 mm. long). . . . *D. caulleryi* n. sp. See Fig. 2, a-o.

The parasitic arabellids may be separated into two main groups. The first includes those species of which numerous specimens are found in a single host. Within a single host, they may be found in varying stages of development, from small specimens, with few segments, no eyes, and no jaws, to larger ones, with numerous segments, eyes, developing jaws, parapodia, and setae. The two reported cases are thought to be the young stages of species of *Notocirrus.* When first observed by Koch (1847), they were reported as a lumbrinerid-like stage of the young of the presumably viviparous eunicid host, *Marphysa sanguinea;* this species lives in a loose mucous tube, irregularly encrusted with rocks, shells, and such. Similar forms have since been found in the onuphid, *Diopatra cuprea,* which lives in a parchment-like tube, one end of which is buried in the sand or mud, the other end sticking out of the substratum and covered with shells, plant debris, etc. The parasites evidently penetrate the host at an early stage, just how early and the mechanism for penetration being unknown. They grow and develop within the host to an advanced stage, when they evidently leave the host and perhaps continue to grow and mature, taking on a free-living existence.

The other type of parasitism is the condition where a single parasite is found in a host and where the parasite may attain enormous dimensions in comparison to the host, becoming nearly as large or larger than the host or host fragment. They evidently penetrate the host at an early stage also and grow to an advanced stage, perhaps even completing their growth within the host. The parasite may be completely enclosed in the host or part of it may protrude. Perhaps it matures after leaving the host, as sex products have not been observed in the parasites found in the host.

The parasitic arabellids appear to be rare. Most of the records of the different species have been based on single specimens. In a long study from 1875 to 1888 at Dinard, Saint-Joseph (1888) observed only 14 examples of *Labrorostratus parasiticus* in the body cavities of several species of syllids. After numerous years of microscopical examination of numerous syllids, Caullery and Mesnil (1916) observed only a single parasite of the same species. Considering that the syllids are small and somewhat transparent and that the parasites may be as much as three-fourths of the length of the host, the presence of the parasites would probably be noted. Where the host is larger and opaque, the parasites would be observed only accidentally in fragmented or dissected specimens.

Four of the parasitic species belong to the genus *Drilonereis,* which also includes free-living species. The maxillae and maxillary carriers are well developed. In *D. caulleryi* n. sp. the parapodial armature develops gradually; first the heavy aciculum appears, then the setae, only the tips of which project at first, then the setae project further and the tips of the heavy acicula finally protrude. In *D. benedicti* n. sp. the acicula and setae are rudimentary and do not project from the parapodial lobes, even in a specimen of more than 1200 crowded segments. The parasitic genera, *Oligognathus* Spengel, *Labrorostratus* Saint-Joseph, and *Haematocleptes* Wirén, have the maxillae more rudimentary than in *Drilonereis,* with a single elongate rodlike maxillary carrier, darker toward the outside. *Haematocleptes terebellidis* shows the most rudimentary condition, having only a single pair of maxillae and the setae and acicula not projecting from the parapodial lobe.

Genus *Drilonereis* Claparède, 1870

Type (by original designation): *D. filum* (Claparède, 1868).
Labidognathus Caullery 1914; type (by monotypy): *L. parasiticus* Caullery, 1914.

Diagnosis. Prostomium conical to spatulate, flattened ventrally, usually with central depression dorsally, without eyes or appendages. First two segments apodous and achaetous, first sometimes partially fused dorsally to prostomium. Parapodia with dorsal lobe or notopodium usually small, rudimentary, with a few embedded notopodial acicula; neuropodium with two unequal lips, supported by acicula. Setae all simple, of two kinds: (1) bilimbate or winged, tapering to fine tips, smooth or faintly striated (not denticled); (2) 1–2 stout acicular setae with tips protruding from parapodial lobe; (in some parasitic forms, setae may be rudimentary, not extending out of parapodial lobes). Pharynx with mandibles or lower jaws rudimentary or absent; maxillae or upper jaws 4–5 pairs, symmetrical, dark, chitinous, supported by a pair of long slender maxillary carriers and a shorter unpaired piece; basal maxillae I large, heavy, falcate pincers or forceps; maxillae II rectangular plates, usually denticled; maxillae III and IV with one to few teeth; maxillae V rudimentary or absent.

Remarks. Labidognathus is herein referred to *Drilonereis.* The type species of the former, *L. parasiticus* Caullery (1914, p. 490), was found living as a parasite in a terebellid (not yet described) near Timor, Dutch East Indies; the parasite was found in the peri-intestinal blood sinus, coiled in a complicated manner around the intestine of the host. According to Caullery, both the host and parasite were in rather poor condition; the jaw apparatus of the parasite was not studied completely. Hartman (1944, p. 180) noted the affinities of *Labidognathus* with *Drilonereis* and described a new species, *L. forcipes,* found in the body cavity of a fragment of a species of *Eunice* from San Benito Island, Mexico. In addition two new species are described below. Because of the scarcity of material of the parasites, it is difficult to work out the developmental stages. As indicated below for the four specimens of *Drilonereis caulleryi* n. sp., parasitic in *Onuphis conchylega,* there are differences in the development of the jaws and parapodia in different stages of growth. The four parasitic species of *Drilonereis* show essentially the characters of the genus. They all lack mandibles and have four pairs of maxillae, of which maxillae I are stout falcate hooks and maxillae III and IV each a single stout conical hook. The parasites were found singly, one to a host. The hosts, at least when collected, were either anterior or middle fragments, with the parasites protruding and exposed in part.

Drilonereis benedicti n. sp.

Fig. 1, A–G

The species is known from a single specimen, incomplete posteriorly (U.S.N.M. No. 28637), found in a fragment of 18 segments from the branchial region of *Onuphis magna* (Andrews), North Channel into Tampa Bay, Florida, 12 fms., *Fish Hawk* Sta. 7108, 1901, Dr. J. E. Benedict, collector. The parapodia of the host fragment were compared with those of an incomplete specimen of *O. magna* found at the same station. The middle part of the parasitic *Drilonereis* extended through the body cavity of the host fragment, the greater part of the anterior and posterior

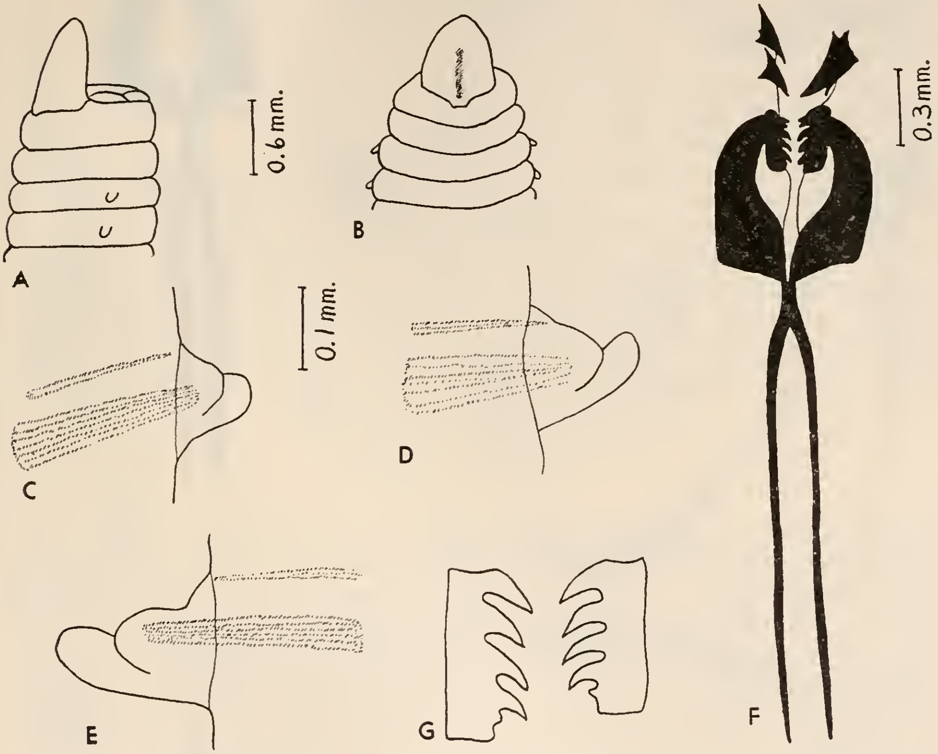


FIGURE 1. *Drilonereis benedicti* n. sp.: A, Lateral view anterior end; B, dorsal view anterior end; C, left parapodium from setiger 10, anterior view; D, same from about setiger 300; E, right parapodium from about setiger 600, anterior view; F, four pairs maxillae and maxillary carriers, dorsal view (more ventral unpaired piece not shown); G, maxillae II, enlarged.

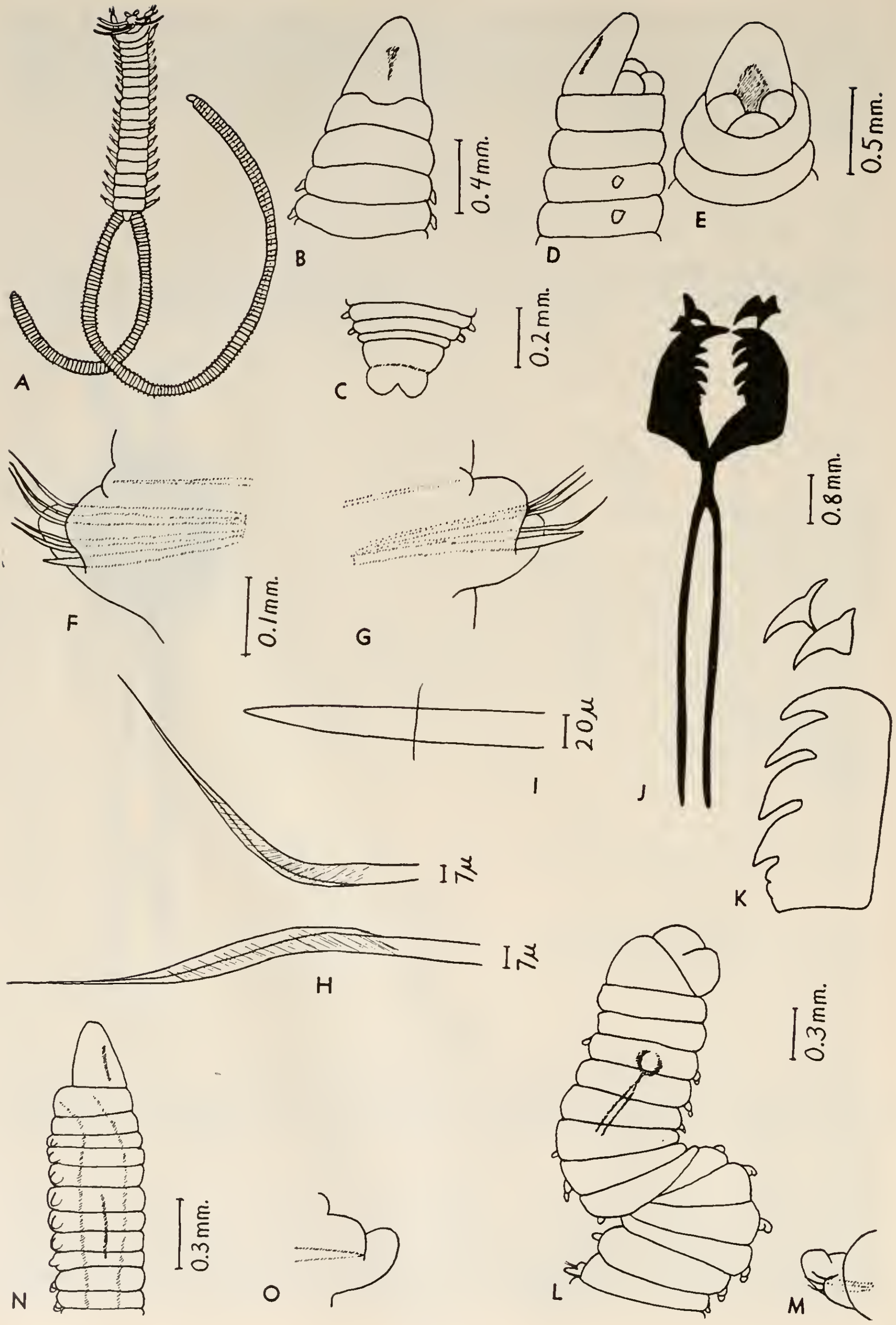

A
B
0.4 mm.
D
E
0.5 mm.
C
0.2 mm.
0.8 mm.
F
0.1 mm.
G
I
20 μ
J
K
7 μ
H
7 μ
0.3 mm.
N
0.3 mm.
O
L
M

FIGURE 2.

ends of the parasite being exposed. It is named for the collector, who evidently put it aside to be worked up later.

Description. Length of incomplete specimen 240 mm., greatest width 1.5 mm., segments more than 1200. Body cylindrical, with segments slightly longer anteriorly becoming very short and crowded posteriorly, colorless (in alcohol), shiny iridescent anteriorly, dull posteriorly. Prostomium (Fig. 1, A, B) conical, rounded anteriorly, flattened ventrally, with a longitudinally depressed area mid-dorsally. First two segments achaetous, subequal to the following, first with mid-dorsal nuchal notch. Parapodia (Fig. 1, C–E) similar along length of body, small, unequally bilobed, with shorter rounded and longer thick digitiform lobes. No setae exposed external to lobes; internally few notopodial acicula extending into base of rudimentary low notopodial lobe and larger group of neuropodial acicula and setae with tips extending into the short neuropodial lobe; one of the acicular group is much stouter, probably corresponding to the stout acicular seta characteristic of *Drilonereis.* Proboscis without mandibles and with four pairs maxillae. Maxillae (Fig. 1, F, G) well developed, dark, with a pair of long, dark filiform maxillary carriers and short oval unpaired piece, dark anteriorly, light amber-colored posteriorly; basal maxillae I stout, falcate, hooked; maxillae II rectangular plates, each with four distinct teeth and slight indication of a fifth; maxillae III and IV each a single, large, thorn-like tooth.

Remarks. D. benedicti differs from the other parasitic species of *Drilonereis* in the complete absence of exposed setae, even in a specimen of more than 1200 segments. The host normally lives in a parchment-like tube. Perhaps the host fragment moves along in the tube carrying the parasite with it. Possibly the parasite at this stage feeds for itself, as the anterior and posterior ends were exposed. There is also the possibility that the host fragmented at the time it was collected.

Drilonereis caulleryi n. sp.

Fig. 2, A–O

The species is represented by four specimens, each of which was found living parasitically in anterior fragments of *Onuphis* (*Nothria*) *conchylega* Sars, collected by the *Fish Hawk* and *Albatross* off Martha's Vineyard, Massachusetts, and off Cape Henry, Virginia, from 1880 to 1883. Two of the four specimens are small, showing different developmental stages. The specimen designated as the type (U.S.N.M. No. 12867) is complete and was found coiled inside a host of about 40 anterior segments. The largest paratype (U.S.N.M. No. 8987) consists of an anterior end of 25 mm. and a posterior end of 10 mm. protruding from the posterior

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FIGURE 2. *Drilonereis caulleryi* n. sp.: A, Habit sketch of parasite and host (2); B, dorsal view anterior end (1); C, dorsal view posterior end (1); D, lateral view anterior end, proboscis partially extended (2); E, ventral view same; F, left parapodium from setiger 20, anterior view (1); G, right parapodium from middle of body, anterior view (1); H, limbate setae from same; I, acicular seta from same; J, four pairs maxillae and maxillary carriers, dorsal view (more ventral unpaired piece not shown) (2); K, right maxillae II–IV, enlarged; L, dorsal view anterior end of slightly coiled smaller paratype, proboscis partially extended (3); M, parapodium from setiger 10 of same; N, slightly lateral view anterior end of smallest paratype (4); O, parapodium from setiger 12 of same. (1) type specimen; (2) largest paratype specimen; (3) smaller paratype specimen; (4) smallest paratype specimen.

end of the host fragment of 18 segments, the middle part of the parasite being inside the host (Fig. 2, A). A smaller paratype (U.S.N.M. No. 28636) occupied setigers 8–18 of an anterior fragment of a host of 18 segments; the posterior end of the parasite protruded from the posterior end of the host fragment. A very small paratype (U.S.N.M. No. 28635) was found in a host fragment of 17 segments; the anterior end of the parasite was sticking out dorsally between setigers 15 and 16, the posterior end was protruding ventrally between the same segments. The species is named for Dr. Maurice Caullery, who described the first parasitic drilonereid (as *Labidognathus*).

Description. Length up to 110 mm., greatest width up to 1 mm., segments up to 400 or more. Body cylindrical, shiny iridescent. Prostomium (Fig. 2, B, D, E) conical, rounded anteriorly, flattened ventrally, with a mid-dorsal depressed area. First two segments apodous and achaetous, subequal to the following, first with mid-dorsal nuchal notch. Anal end (Fig. 2, C) short, cylindrical, tapering to pair of short bulbous lobes (no distinct anal cirri). Parapodia (Fig. 2, F, G) similar along length of body, short, unequally bilobed, with shorter rounded and longer, thick digitiform lobes. Two larger specimens with two kinds of setae projecting from parapodial lobe: 4–5 bilimbate, straight and curved setae, tapering to slender tips, faintly striated (not denticled; Fig. 2, H); also single yellowish stout, pointed acicular seta (Fig. 2, I); with additional internal neuropodial acicula as well as few notopodial acicula, the tips of which extend into short bulbous rudimentary notopodium; posterior end of body with tips of limbate setae only projecting from parapodial lobes. Smaller specimen (12+ mm. long, 0.6 mm. wide) with tips of limbate setae only extending out of lobe, stout acicular seta being visible inside lobe (Fig. 2, 1, M). Smallest specimen (5 + mm. long, 0.3 mm. wide) with no setae projecting, single stout acicular seta being visible inside parapodial lobe (Fig. 2, N, O). Proboscis, when partially extended, appearing as three bulbous lobes (Fig. 3, D, E); no mandibles; maxillae four pairs, well developed, dark, with pair of long filiform carriers and shorter wide oval unpaired piece, dark anteriorly, lighter more posteriorly (Fig. 2, J, K); maxillae I stout, falcate, forceps-like; maxillae II rectangular plates, each with four distinct teeth; maxillae III and IV each a single large hooked thorn-like tooth. In smaller specimen, maxillary forceps and slender carriers visible through transparent body wall (Fig. 2, L). In smallest specimen (Fig. 2, N) only maxillary carriers visible.

Remarks. D. caulleryi differs from the other parasitic species of *Drilonereis* as indicated in the key above. As shown in the three developmental stages found in the four specimens, the maxillary carriers develop first, then the stout maxillary forceps; in the parapodia, the stout acicular seta appears first, then the limbate setae, the tips of which may protrude, the stout acicular seta not protruding (this would correspond to the condition described for *D. forcipes* by Hartman); finally the limbate setae protrude further and the tips of the stout acicular setae protrude. The host, *Onuphis conchylega* Sars, occupies a flat parchment-like tube encrusted with flattened pieces of rocks and shells. All four host specimens are anterior fragments. The parasite may be completely enclosed in the host fragment or a portion of the parasite may stick out, revealing its presence. The parasite perhaps gets into the host by encouraging it to fragment; none of the fragments showed any signs of regeneration, although *O. conchylega* fragments and regenerates readily.

Material examined. Type: off Martha's Vineyard, Massachusetts, 39° 53′ N., 69° 47′ W., 317 fms., soft green mud, *Fish Hawk* Sta. 1096, 1882. Paratypes: 40° 02′ N., 70° 23′ W., 115 fms., mud, fine sand, *Fish Hawk* Sta. 871, 1880; 40° 02′ N., 70° 37′ W., 101 fms., grey mud, fine sand, *Fish Hawk* Sta. 1108, 1882; off Cape Henry, Virginia, 37° 19′ N., 74° 26′ W., 102 fms., green mud, shell, *Albatross* Sta. 2004, 1883.

Distribution. Atlantic, off Massachusetts to off Virginia. 101 to 317 fathoms.

Genus *Notocirrus* Schmarda, 1861, emend. Ehlers, 1868

Type (designated by Ehlers, 1868, p. 406) : *N. chilensis* Schmarda, 1861.

Diagnosis. Prostomium conical, without appendages, usually with 4 eyespots. First two segments apodous and achaetous. Parapodia with dorsal lobe or notopodium represented by small rudimentary lobe and few notopodial acicula; neuropodium unequally bilobed, supported by stout acicula, the tips of which project (except in young developing stages). Setae all simple, bilimbate or winged, tapering to fine tips, striated and finely to coarsely denticled along limbate border. Pharynx with pair of wing-shaped, dark chitinous mandibles or lower jaws; maxillae or upper jaws 4–5 pairs, dark, chitinous, denticled, supported by pair of long filiform maxillary carriers and shorter unpaired piece; maxillae I and II asymmetrical, maxillae I dentate throughout entire length or only slightly falcate or with short hook distally.

Remarks. *Notocirrus* has affinities with *Arabella,* having similar prostomia and pharyngeal jaws; both have limbate setae with denticled border. *Notocirrus* also has affinities with *Drilonereis,* both having stout acicula or acicular setae which project from the parapodial lobes. A species of *Notocirrus* is herein reported to be parasitic in an onuphid (*Diopatra*) during its early development.

Notocirrus spiniferus (Moore, 1906)

Figs. 3–5

Arabella spinifera Moore, 1906, p. 501, pl. 19, figs. 1–7.

The species was described from a single specimen which was dredged on muddy bottom in the middle of Buzzards Bay, Massachusetts. No additional records have been reported. In working over a good deal of material collected in New England and vicinity, four additional free-living specimens were found. The species appears to be rare but, due to its superficial resemblance to the more common *Arabella iricolor,* it may be confused with that species in collections. The four specimens were obtained from the following sources: Buzzards Bay, Massachusetts, 1956, H. Sanders, collector; off Cape Henry, Virginia, Chesapeake Bay, 9 fms., shelly and sand, *Fish Hawk* Sta. 8838, 1920; Isle of Wight Bay above Ocean City, Maryland, 1953, S. McDowell, collector; Beaufort, North Carolina, 1951, E. Cole, collector.

In addition two of the specimens found living parasitically in the body cavity of *Diopatra cuprea* (Bosc), collected by M. Jean Allen at Hadley Harbor, Nonamesset Island, Woods Hole region, Massachusetts, were examined. They ap-

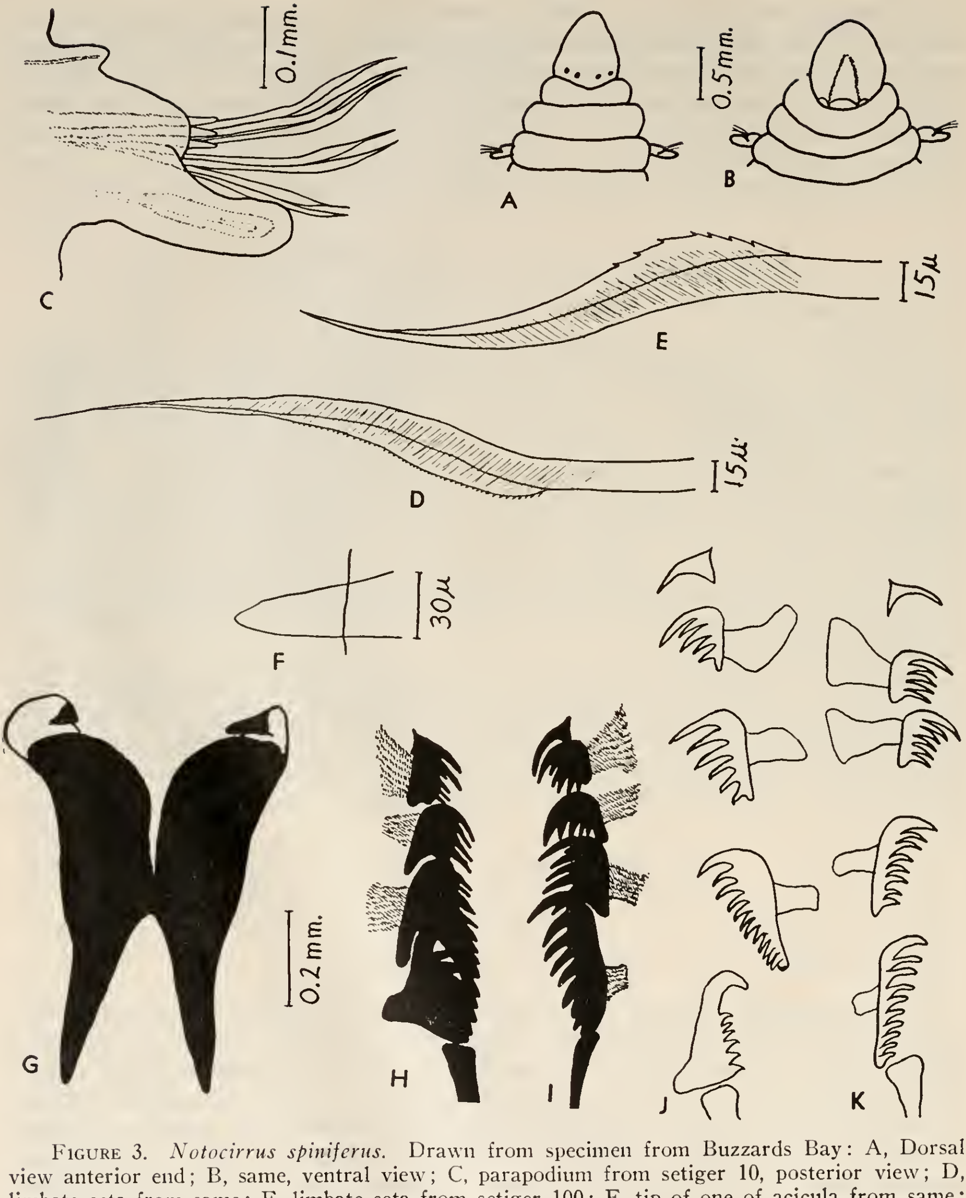

FIGURE 3. *Notocirrus spiniferus.* Drawn from specimen from Buzzards Bay: A, Dorsal view anterior end; B, same, ventral view; C, parapodium from setiger 10, posterior view; D, limbate seta from same; E, limbate seta from setiger 100; F, tip of one of acicula from same; G, mandibles, ventral view; H, left maxillae, I–IV, dorsal view; I, right maxillae, I–IV, dorsal view; J, left maxillae spread apart; K, right maxillae spread apart.

pear to be the young of a *Notocirrus*, possibly that of *N. spiniferus*. In a note recording the find of more than 50 parasitic specimens in a single specimen of *Diopatra,* Allen (1952) indicated that they might be the young of *Arabella iricolor* but the parapodia differ from that species as indicated below.

Description of specimens found free-living. Length 40–110+ mm., width 1–4 mm., segments 140–220+. Body cylindrical, tapering slightly anteriorly and posteriorly, stiff, wiry. Prostomium (Figs. 3, A, B; 4, A, B) subconical, rounded anteriorly, slightly depressed dorsoventrally but not greatly flattened as in *Drilonereis;* a pair of faint longitudinal grooves ventrally and four eyes in transverse row at posterodorsal border, rather than two eyes as reported by Moore. First two segments achaetous and apodous, first with mid-dorsal nuchal notch. Parapodia (Figs. 3, C; 4, D) prominent, similar along length of body, with small but distinct notopodium supported internally by few notoacicula; neuropodium bilobed, with short rounded setal lobe and longer, prominent digitiform postsetal lobe (sometimes referred to as ventral cirrus or cirriform branchial organ), within which is a vascular loop. Setal lobe with 1–3, usually 2, stout, deep yellow acicula, the tips of which project out of lobe (thus differing from *Arabella,* which has no stout projecting acicula); acicular tips bluntly pointed, tapered abruptly to short fine tips, or sometimes obviously broken (Fig. 3, F). Setal lobe with 4–8 bilimbate, doubly curved setae with fine tips, with wings wide, striated and finely denticled along border, sometimes with a few coarser denticles near base of wing (Fig. 3, D, E). Anal end (Fig. 4, C) tapered to pair of short bulbous lobes, each with very short rudimentary anal cirrus.

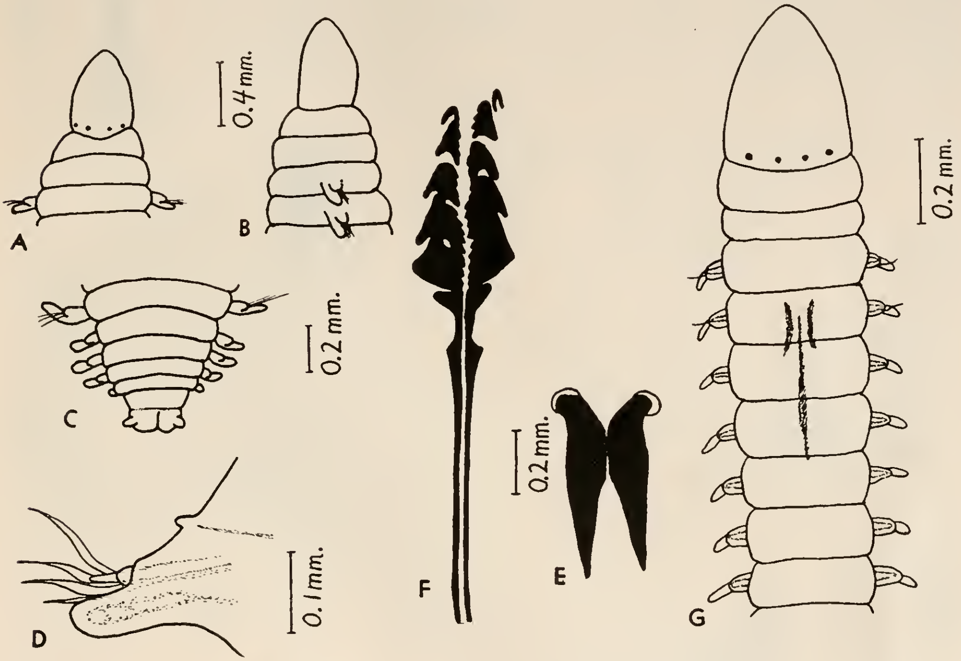

FIGURE 4. *Notocirrus spiniferus.* Drawn from specimen from Isle of Wight Bay (A–F): A, Dorsal view anterior end; B, lateral view same; C, dorsal view posterior end; D, parapodium from middle of body; E, mandibles, ventral view; F, five pairs maxillae and maxillary carriers, dorsal view (more ventral unpaired piece not shown). G, Dorsal view anterior end of small specimen of *Notocirrus ?spiniferus,* living parasitically in *Diopatra cuprea,* Woods Hole region.

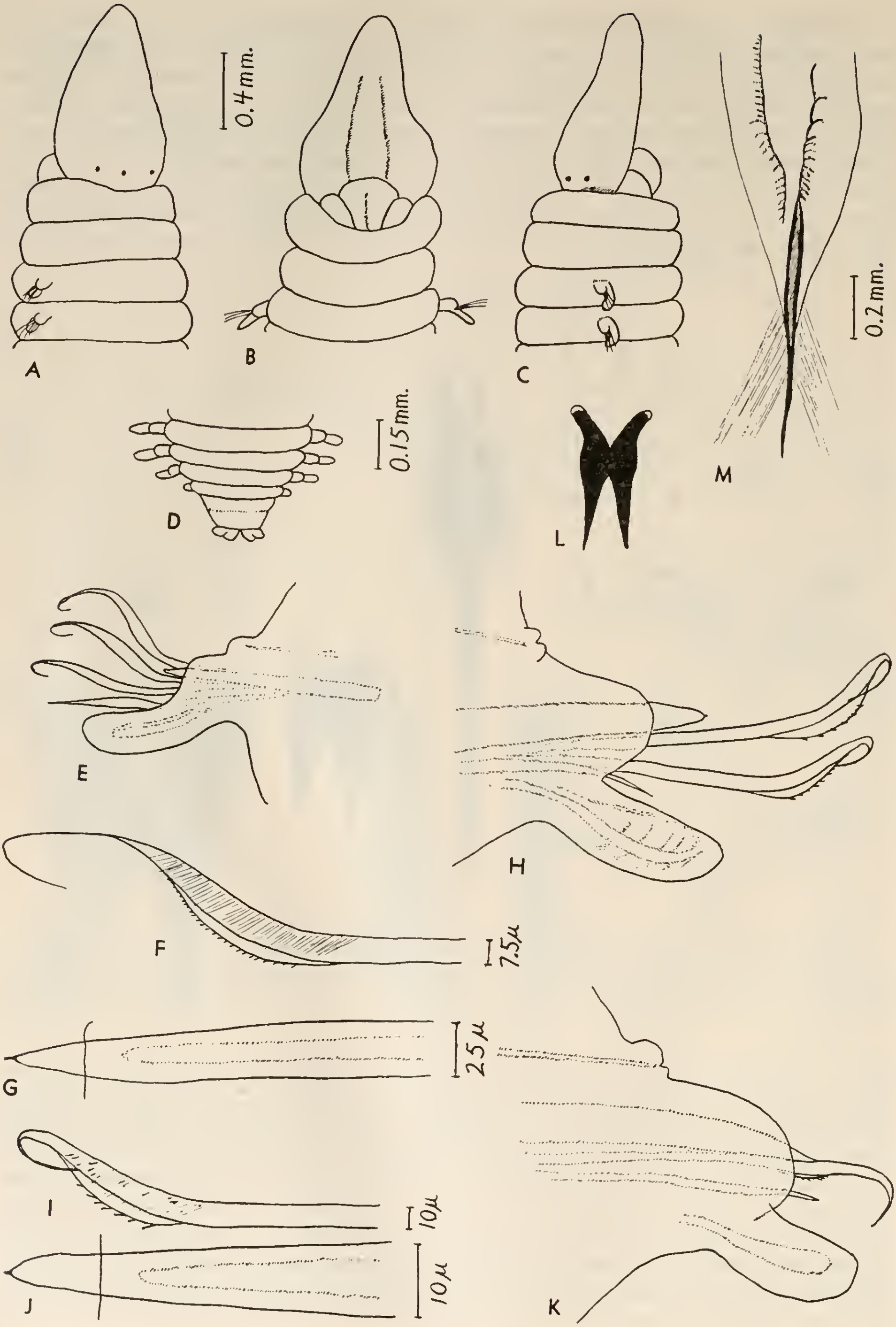
0.4 mm.
A
B
C
D
0.15 mm.
L
M
0.2 mm.
E
H
F
7.5μ
G
25μ
I
10μ
J
10μ
K

FIGURE 5.

Proboscis, when partially extended, appears as bulbous bilobed tongue with pair of lateral lobes. Ventral mandibles (Figs. 3, G; 4, E) well developed, brown or black, wing-shaped, sometimes with exposed white tips. Five pairs of dorsal jaws or maxillae (Figs. 3, H–K; 4, F), with pair of long slender carriers, thickened distally and subdistally, and shorter unpaired piece; fifth pair, consisting of single tooth, easily confused with the fourth, to which it is closely allied. First two or basal pairs of maxillae asymmetrical. Right maxilla I longer than left, with up to 10 denticles along length of inner border and without distal hook; left maxilla I with up to six basal teeth and distinct distal hook. (Moore, in figure of the type, showed distal hooks on both first maxillae and fewer teeth.) Left maxilla II much larger than right, completely overlapping left maxilla I and extending down to maxillary carriers, with up to 12 or 13 teeth; right maxilla II partially overlapping right maxilla I, with up to 8 or 10 teeth. Maxillae III to V symmetrical, III each with 6 teeth, IV each with 5 teeth, and V each a single tooth, without basal extension as in other maxillae. (The last may appear to blend in with maxillae IV and this may have led to Moore's count of only four pairs of maxillae.) Color (in alcohol) yellowish to brownish, iridescent.

Description of two young specimens of Notocirrus ?spiniferus, living parasitically in Diopatra cuprea. The larger parasitic specimen is 25 mm. long, 1.5 mm. wide, and consists of almost 200 segments. The smaller specimen is 12 mm. long, 0.5 mm. wide, and contains about 150 segments. Prostomium of smaller one (Fig. 4, G) conical, bullet-shaped, that of larger one (Fig. 5, A–C) more elongated, bulbous basally; both show four eyes in transverse row. Parapodia of smaller specimen showing characteristic bilobed form, without setae projecting except for first two setigerous segments in which a single seta projects; each of setal lobes provided with stout aciculum (appears dark basally). In larger specimen, parapodia of anterior region (Fig. 5, E) with four limbate setae and single stout aciculum; those of middle region (Fig. 5, H) with two limbate setae and tip only of a third one projecting; those of posterior region (Fig. 5, K) with single limbate seta and tip of a second one projecting; notopodia small but distinct, with tip of notaciculum extending into lobe. Limbate setae with long fine tip, curled backward, distinctly denticled along limbate border (Fig. 5, F, I); stout projecting acicula with fine short tips (Fig. 5, G, J). Thus parapodia essentially as in larger free-living specimens of *Notocirrus spiniferus* except for fewer acicula and setae. Anal end of small specimen tapering gradually to cylindrical posterior end, without distinct anal cirri; that of larger specimen (Fig. 5, D) essentially as in *N. spiniferus*. Pharynx, when partially extended, appears as three-lobed structure, the middle lobe rounded, tongue-like, consisting of pair of lobes (Fig. 5, B). Jaw parts of smaller specimen (Fig. 4, G) not dissected but darker paired mandibles and maxillary carriers visible through the somewhat transparent body wall, maxillary carriers appearing as single elongated rod. Jaw parts of larger specimen, when dissected out, showing well

FIGURE 5. Young *Notocirrus ?spiniferus*, parasite taken from body cavity of *Diopatra cuprea*, Woods Hole region: A, Laterodorsal view anterior end; B, ventral view same; C, lateral view same; D, dorsal view posterior end; E, parapodium from setiger 10; F, limbate seta from same; G, aciculum with projecting tip from same; H, parapodium from middle of body; I, limbate seta from same; J, aciculum with projecting tip from same; K, parapodium from posterior part of body; L, mandibles, ventral view; M, developing maxillary carriers and maxillae, dorsal view.

developed mandibles (Fig. 5, L), and incompletely developed maxillae (Fig. 5, M) with elongated rodlike maxillary carriers (appearing as single rod but darker toward the outside), and developing denticled maxillae, indicated by slightly darker amber-colored areas on walls of pharynx.

Remarks. The smallest parasites reported by Allen were composed of seven segments; some specimens of 30 segments showed no eyes or setae; the largest specimen reported was 50 mm. long and was composed of about 200 segments. These parasites had not emerged naturally from the host. Thus they live a parasitic life for a considerable period. The smaller specimens were in the body wall of the host near the parietal blood vessels, the larger ones were free in the body cavity. A few large parasites were observed emerging from the body cavity of *Diopatra* by Dr. Frank Brown (Allen, 1952), but the lengths and the developmental stages of these specimens were not indicated. It may be that the parasites remain in *Diopatra* until the jaw parts are completely developed; they were not completely developed in the largest specimen I was able to examine. It is unknown how the parasites get into the host. Perhaps the eggs of *Notocirrus* are laid and fertilized within the tube of *Diopatra. Notocirrus,* being a burrowing form, could enter the parchment-like tube of *Diopatra* on the buried end which, as far as has been observed, is open. In some way, the fertilized eggs or young at a very early stage get into the body cavity of *Diopatra.* The posterior end of *Diopatra* is soft and flaccid and fragments easily; perhaps the young are able to bore into the broken fragmented end of the host. *Diopatra* regenerates readily also. One host, found by Allen, contained about 30 small parasites, composed of from 7 to approximately 30 segments; another host contained over 50 parasites of varying sizes, some up to 50 mm. in length.

In this connection, it may be of interest to mention the observation made by Koch (1847) of filamentous lumbrinerid-like forms crawling out of a broken truncated posterior end of a strongly contracting specimen of *Marphysa sanguinea,* which was dredged at considerable depth in the Mediterranean. On further examination of the *Marphysa,* he found numerous young specimens in the body cavity, in various stages of development; the smallest were small roundish microscopic forms with only slight indication of a few segments and without eyes; a more advanced stage of 25–30 segments showed a distinct prostomium with 2 eyes, parapodia with stout aciculum only, and jaw apparatus in early stages of development; a still more advanced stage of 50–100 segments showed a distinct prostomium with 4 eyes in a transverse line, parapodia with 2 stout acicula and a few setae confined to the parapodia; a still later stage of 100–120 segments showed the parapodia with a short rounded setal lobe and a longer postsetal lobe, with stout dark yellow acicula, the tips of which appear from Koch's figure to project, as in *Notocirrus* or *Drilonereis,* and the jaw apparatus well formed (probably not completely formed). Koch thought that the specimens he observed were the young stages of a viviparous *Marphysa sanguinea* and that during its development, the young pass through a lumbrinerid-like stage. Ehlers (1868, p. 364), commenting on the observation of Koch, indicated that Koch had more likely observed parasitic forms of a lumbrinerid-like species living in *M. sanguinea* and that the latter was not viviparous; he stated that it was unreasonable to think that a young specimen of *M. sanguinea* of more than 100 segments would not show some of the characteristics of the adult, that of antennae, branchiae, characteristic setae, etc. In consideration of the long jaw pieces (maxillary carriers) and the four eyes, Ehlers concluded that it might be a parasitic species of *Arabella*. The figures and description given by Koch suggest to me that the young developing stages in *Marphysa* that he observed, were the parasitic young stages of a species of *Notocirrus*, as indicated especially by the long maxillary carriers, the stout parapodial acicula, and the four eyes in a transverse row.

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A DIURNAL ACTIVITY RHYTHM IN PLETHODON CINEREUS AND ITS MODIFICATION BY AN INFLUENCE HAVING A LUNAR FREQUENCY

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It has become increasingly clear that many of the physiological processes in organisms do not occur at constant rates, even when the organism is in a constant laboratory environment. These fluctuations in rates are often of regular recurrence and may be designated as rhythms.[2] Various manifestations of these changes taking place within the organisms may be observed. Among them are rhythms of O_2-consumption and CO_2-production, locomotor activity, chromatophore pigment dispersal and body temperature changes.

Judging from the number of contributed works in the field of biological rhythms, locomotor activity has been more often utilized as an index to rhythmic behavior than any other kind of biological process. The simplicity of automatic recording devices needed, the long span of time over which animals may be used for such studies, and the minimal interference with the animals' normal functioning, are some of the reasons why activity studies have been so popular.

Among the earliest investigators of activity rhythms was Szymanski (1918), who demonstrated them in a variety of animals. Subsequently, studies of the activity rhythms of a host of animals have been made. Among them, to name only a few representative ones, are those of Ralph (1957) on the earthworm, Harker (1956) on *Periplaneta americana,* Brown (1954) on the oyster, Marx and Kayser (1949) on lizards, and Aschoff (1952) on mice.

The diurnal rhythm is the one most commonly encountered in terrestrial organisms (reviewed by Welsh, 1938, and Kleitman, 1949). Among marine organisms both diurnal and tidal rhythms have been found associated together (Brown, Fingerman, Sandeen and Webb, 1953), but sometimes a tidal rhythm may be the only one apparent (Rao, 1954). Lunar periodicities in marine animals are well known (Korringa, 1947).

That lunar influences may also be significant in the metabolic rhythms of terrestrial organisms has been indicated by recent works (Brown, Freeland and Ralph, 1955; Ralph, 1957). The present study was undertaken in order to examine the activity behavior of a terrestrial animal, the salamander, *Plethodon cinereus,* and to analyze it for lunar influences.

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[2] A rhythm, as used here, is defined as a definitely persisting, regularly recurring, quantitative change that continues after external stimuli are withdrawn. Rhythms can be roughly divided into (a) those of 24 hours (diurnal) or less, and (b) those of more than 24 hours (*e.g.,* lunar). They generally have a causal relation to external factors, but are to be distinguished from periodicities which are of extrinsic origin and which vary directly with environmental factors.

EXPERIMENTAL PROCEDURE

Twenty-four adult *Plethodon cinereus* were collected on May 8, 1955, in a beech-maple forest near New Buffalo, Michigan. The animals were brought to the laboratory and placed in two 9-inch crystallizing dishes, the bottoms of which were covered with a moist sand layer and bits of wood debris. Small earthworms and sowbugs were provided as food and these were replenished frequently during the experiment. The dishes were placed in a slowly-moving stream of cold tap water.

All work was carried out in a room designed as a photographic darkroom. There was one opening to the room, a door fitted with a light-baffle. Thermograph records of the air temperature a few inches above the running water in which the animals were kept throughout the experiment were taken between May 10th and 26th. These showed no diurnal temperature variations, but rather only slow changes requiring several days.

Continuous records of the locomotor activity of seven animals were obtained by the use of the same apparatus, and data were translated from the experimental records to tabular form in the same manner, as described by Ralph (1957) for the study of the earthworm. The recording device consisted essentially of seven rectangular platforms that rocked freely upon knife-blade bearings. Upon each platform was mounted a chamber consisting of a 3¾-inch Petri dish enclosing the bottom half of a 2¾-inch Petri dish, thereby forming a circular track one-half inch in width. Movement of the animal around this track resulted in different degrees of tipping of the platform. Platform movements were transmitted via a thread to a recording pen system that reproduced them on a sheet of paper moving at a rate of two centimeters per hour. The recording apparatus occupied a position adjacent to the dish of reserve animals and the activity chambers were suspended about two inches above the water's surface in order to keep the animals in the same cool air layer.

From May 9th to 13th all the animals were exposed to alternating light, from 6 A.M. to 6 P.M., and darkness, from 6 P.M. to 6 A.M. The light source during this period was two 7½-watt opalescent, incandescent lamps suspended about four feet above the animals. The form of the diurnal activity cycle under simulated day and night conditions was determined during this period.

During the afternoon of May 13th a light-proof box was mounted over the water table in which the reserve animals were kept so that they could be maintained in constant low illumination. The box was equipped with a light source at the top so that diffuse light of less than one foot-candle reached the salamanders. Also, the transparent glass covers of the activity chambers were replaced by black-painted covers in order to exclude all light. Thus, when the animals were in the reserve dishes they were under continuous and constant low illumination, and when placed in the recording apparatus they were in darkness. Twenty-nine days of continuous records for seven animals at a time were obtained under these conditions.

A regular sequence of replacement was established at the outset of the experiment, so that only one or two animals were replaced daily. As the animals were removed from the chambers they were placed in one of the crystallizing dishes, while the replacement animal was randomly selected from the other dish. When the supply of animals was exhausted from one dish, replacement was started from the dish that had been receiving the animals. Thus, each of the 24 animals participated in the study at least twice for approximately four days each time.

In order to minimize any effects that the placing of a fresh animal in the apparatus might have on the data, the time of replacement was varied over the day from 8 A.M. to midnight. As a further precaution, the first three hours of the record produced by a fresh animal were not included in the data, since the animals tended to be hyperactive for several minutes after being placed in a chamber.

RESULTS

Diurnal rhythm

The mean activity for the animals in alternating light and darkness is shown by Figure 1, A.[3] It will be seen that the animals were most active during the dark hours and least active during the light hours. A more complete analysis of the exact form of this curve will be presented in the discussion.

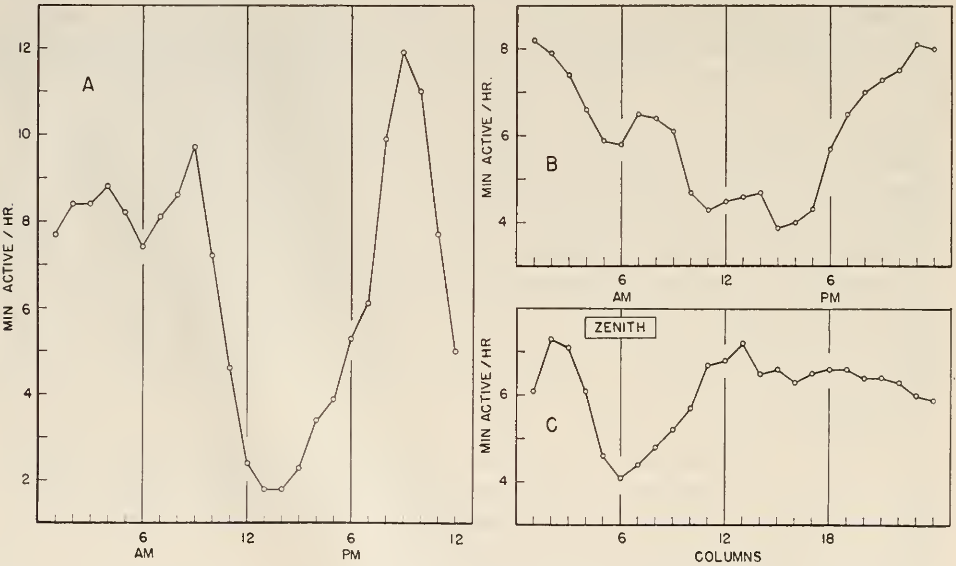

FIGURE 1. (A) The average activity of seven salamanders for about five consecutive days while exposed to light from 6 A.M. to 6 P.M. and darkness from 6 P.M. to 6 A.M. (B) The average activity of seven salamanders for 29 consecutive days. Their activity was recorded while they were in darkness. (C) The influence of the lunar-day frequency on the activity of the salamander, as demonstrated by analysis for lunar effects. (See text for explanation.) The times of zenith for the 29-day period were synchronized in column 6 for this analysis.

Table I shows the mean activity when the animals were in dark chambers. These data represent hourly determinations for 29 consecutive days. The mean values for seven animals for each hour were placed in the table under the hour on which the determination ended.

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[3] All graphs in this paper are plotted from sliding averages of three adjacent values. For example, the averages of the 2, 3, and 4 P. M. columns were averaged to give the 3 P.M. value. This technique is useful for smoothing curves and shows trends more clearly.

Table I

Locomotor activity in the salamander (min./hr.)

	A.M.												P.M.											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
May 14	3.6	1.9	7.9	1.9	0	0.3	5.7	5.7	6.0	1.1	6.7	5.0	4.0	0	0.7	1.4	1.4	5.6	1.0	8.6	12.9	8.6	6.4	0.3
15	0.4	5.0	9.6	0.3	0.3	5.7	0.3	0.4	4.3	0.7	6.7	5.0	0.3	0.3	0.7	3.6	6.0	12.1	15.7	22.4	5.7	11.7	3.6	7.4
16	10.7	11.0	7.1	5.0	11.1	7.1	0	0.6	1.7	0.3	1.4	1.4	6.1	6.2	3.3	7.0	7.0	8.5	5.8	0.7	8.6	6.7	11.0	22.1
17	10.7	8.6	4.9	7.9	2.1	3.1	0	4.3	5.0	4.6	0.8	4.6	1.4	0	0.3	0	2.1	0.4	3.6	5.7	1.4	0	7.1	7.9
18	4.6	5.0	8.6	7.4	0	5.0	8.9	1.0	4.3	4.3	2.4	0.3	8.6	10.0	3.3	1.0	0	0	16.7	9.7	5.3	2.4	8.9	8.6
19	8.4	11.0	8.6	0	2.1	2.9	0	7.1	1.4	0	3.7	0	0	0	0.7	0	2.0	7.0	1.5	4.3	5.0	9.3	8.0	4.0
20	4.3	1.9	4.4	12.9	5.3	7.9	16.4	8.1	1.7	2.9	0.7	8.6	1.4	0.3	0	0	0.7	7.1	5.3	8.6	0.4	0	1.9	13.6
21	9.3	7.9	5.0	8.6	12.9	13.6	9.3	27.9	16.4	10.0	0	0.4	8.6	7.9	0	0	0	0	0	0	4.6	10.0	8.9	10.7
22	8.9	15.0	17.0	8.6	11.4	9.3	2.9	3.6	3.6	0.3	4.5	0.8	0.5	5.7	1.4	0.7	0	6.4	6.4	9.3	11.1	8.6	7.1	16.0
23	12.1	7.9	8.6	2.1	2.4	6.0	0.7	11.0	10.0	7.5	4.2	9.3	2.6	2.9	7.9	5.8	0.3	0.7	5.7	7.6	12.5	1.2	0.7	0
24	4.1	0	4.3	9.3	5.0	7.4	9.4	0.6	6.4	0.4	0.3	2.0	0	8.3	10.7	2.1	0.6	2.4	17.4	13.2	4.3	12.1	11.4	0
25	0	1.7	3.6	8.6	5.7	5.0	10.7	4.4	0	6.4	0.3	4.3	0	0	0	0	8.6	8.6	15.3	10.3	18.3	25.0	11.0	19.3
26	17.4	19.3	6.4	0.4	13.6	14.6	4.3	0	0.7	0.3	12.5	14.2	0	2.9	7.9	0.7	16.6	20.0	20.3	17.9	23.1	20.0	15.0	23.6
27	4.3	0	3.1	12.9	14.3	8.9	8.9	0.3	6.4	0	0	3.1	12.5	10.0	6.7	8.9	12.1	1.4	13.6	15.3	2.1	12.5	4.2	10.8
28	16.4	13.2	7.1	2.4	6.7	0	0.3	0	0.7	1.4	1.0	5.0	9.3	9.3	12.9	10.0	0	0	19.2	16.7	14.6	12.1	6.9	5.3
29	13.9	31.4	18.1	12.9	8.6	7.7	5.0	14.3	11.3	8.0	3.3	7.9	10.0	0.7	4.3	8.6	1.4	7.1	5.7	10.0	7.9	17.1	5.0	10.7
30	13.6	2.1	6.1	8.6	11.4	4.6	0	8.6	7.1	7.9	4.3	18.3	17.5	2.8	0	0	0	0	1.4	0.7	0	0.8	0	11.6
31	17.1	13.9	2.1	10.7	7.1	3.8	8.6	16.4	5.7	0.7	5.0	13.6	6.0	3.8	12.1	2.1	15.0	20.0	7.5	7.9	0	0	0	0
June 1	4.2	12.9	12.1	4.3	3.8	2.1	7.1	13.9	11.7	13.9	5.0	1.4	1.4	2.1	5.3	0	5.7	3.6	0	0	2.9	2.8	11.7	10.0
2	3.3	0	13.6	8.6	1.4	0	14.3	11.4	10.3	14.3	2.9	3.6	7.9	3.3	17.5	10.3	11.4	8.6	5.3	0.7	3.6	3.6	0	5.0
3	5.7	8.1	10.7	6.4	9.6	2.1	4.3	15.7	6.4	0	0	0	13.6	10.0	2.9	0	0	2.9	8.9	1.7	0	0	4.2	5.0
4	0	19.3	11.4	2.9	2.1	2.9	13.6	17.1	18.0	18.3	8.8	2.0	0	0	7.1	7.1	13.6	14.3	9.3	8.6	3.6	0	5.7	0
5	4.3	5.3	0	0	7.1	22.9	12.1	14.3	2.1	5.3	8.6	8.6	5.3	8.3	5.3	0	0	0.3	0	2.1	25.6	16.2	4.2	8.6
6	4.6	2,1	6.4	1.4	4.6	6.4	4.3	17.9	10.7	9.1	2.9	3.3	0.3	0	5.7	0	0.6	0	0	0	3.6	8.6	9.6	9.5
7	3.3	10.0	14.3	9.3	3.6	3.6	0	0	0	5.3	9.3	8.6	18.6	10.0	10.8	10.0	15.0	21.4	8.6	0.8	0	9.2	15.0	6.0
8	8.6	3.6	0	0	5.7	18.6	12.1	16.4	5.7	6.4	0	1.4	9.3	3.6	5.8	2.8	0	0	0	2.1	7.9	18.6	15.0	1.4
9	2.1	0.7	12.9	8.6	5.7	0.7	0.7	4.3	3.8	0	0	0.3	1.0	9.5	5.8	0	1.2	0	6.7	0	2.1	0.7	18.9	18.6
10	4.6	7.1	15.0	10.0	3.6	0.3	3.6	3.0	3.1	6.2	3.7	0.2	3.0	1.0	0.3	0.9	4.3	2.9	2.9	6.4	13.6	10.0	17.5	12.8
11	15.0	11.4	5.7	0.7	2.1	2.1	1.9	0.3	0	3.6	6.4	0.7	0.3	0.2	1.0	0.3	3.1	0	5.7	7.1	5.7	1.4	0	11.4

The data were first analyzed to find the mean activity for each hour of the day. All 24 columns were averaged and the results are plotted in Figure 1, B. The form of this variation is similar, in a general way, to that obtained when the animals were exposed to alternating light and darkness. There are conspicuous differences between them, however, and these will be discussed later.

Lunar analysis

The data of Table I were inspected for a lunar influence before they were subjected to a detailed analysis. First the data for each day were plotted, each day under the preceding one, and then a line representing lunar zenith was drawn across the plots, intersecting the abscissae at the time of zenith. Since the moon reaches zenith for any given locality approximately 50 minutes later each day, the line representing zenith intersected the time scales about 50 minutes later with each successive day.

Upon close examination of these plots, it was seen that the activity pattern for each day had certain unique variations, but generally bore a similarity to the mean pattern for the 29 days. However, it was noted that when zenith occurred at times of usually moderate or high activity the level of activity was generally low for a few hours before and after the time of zenith. That is to say, when zenith occurred during the "night hours," which were usually the times of greatest activity, depression of activity resulted.

On the starting day of this 29-day study, May 14th, the moon was in third, or last, quarter and thus the time of zenith was approximately 6 A.M. On May 21st new moon occurred with the time of zenith at noon. First quarter, with zenith at approximately 6 P.M., was on the 28th of May and full moon, with zenith at midnight, occurred on June 5th.

It may be postulated that the mean activity for any one day would tend to be high if zenith occurred during the times of normally low activity and would tend to be lower if zenith occurred during the times of high activity. Therefore, if one plots the mean activity for each day for one lunar month, the resulting curve should be essentially the inverse of the mean hourly activity curve, provided the lunar effect is operative in the postulated manner. A comparison of Figure 2, which shows the daily means for a lunar month, with Figures 1, A or 1, B, the hourly mean curves, bears out this hypothesis. This would then suggest strongly that a lunar zenith effect is operative; it is a depressive effect, and the time at which zenith occurs does indeed appear to determine to some extent the mean activity for any one day.

A second method of demonstrating the presence of an apparent lunar modification of the diurnal rhythm was applied in the following manner. If the hourly averages of the days between third quarter and new moon are found, a pattern of activity variation essentially like the average for the entire 29 days should result, since little depressive influence should be in evidence during this time. Figure 3, A, shows the means for each hour of the day for the seven days of that period, and it will be seen that this pattern is very similar to that for the entire 29-day period. Likewise, the hourly means for the succeeding seven days, May 21st to 27th, that is, from new moon to first quarter, should also be similar to the means for the entire 29 days and similar to the pattern for the preceding seven days. Upon examination of Figure 3, B, it will be seen that this is true.

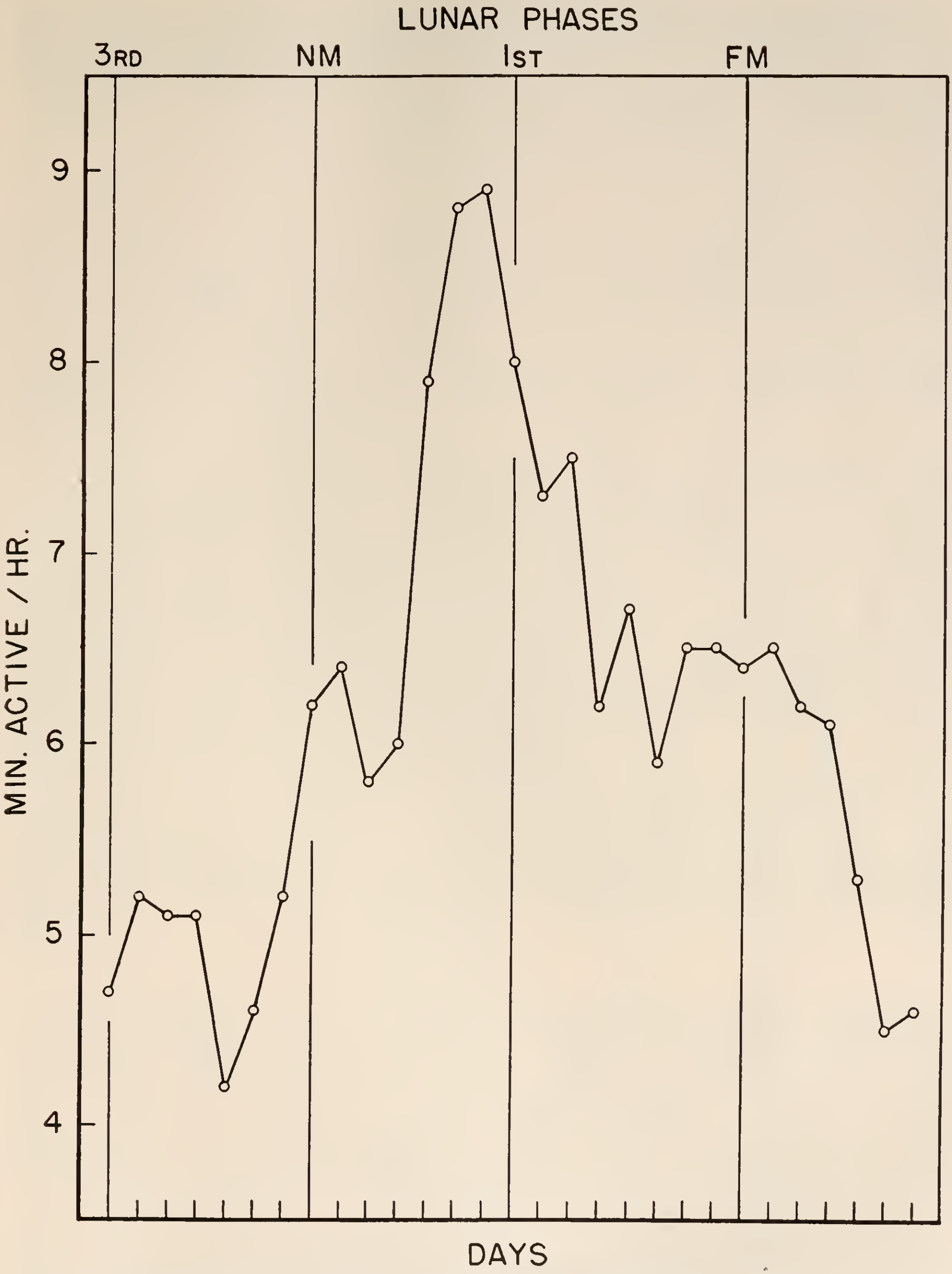

FIGURE 2. The average activity per day for seven salamanders over a 29-day period. The approximate times of the lunar quarters are indicated.

During the next eight days, however, the time of zenith moved from about 6 P.M. to near midnight. Any zenith-associated depressive influence should be very evident during this period. Figure 3, C shows that for these days the lowest levels of activity occurred between 6 P.M. and midnight. Evident, also, in this figure is

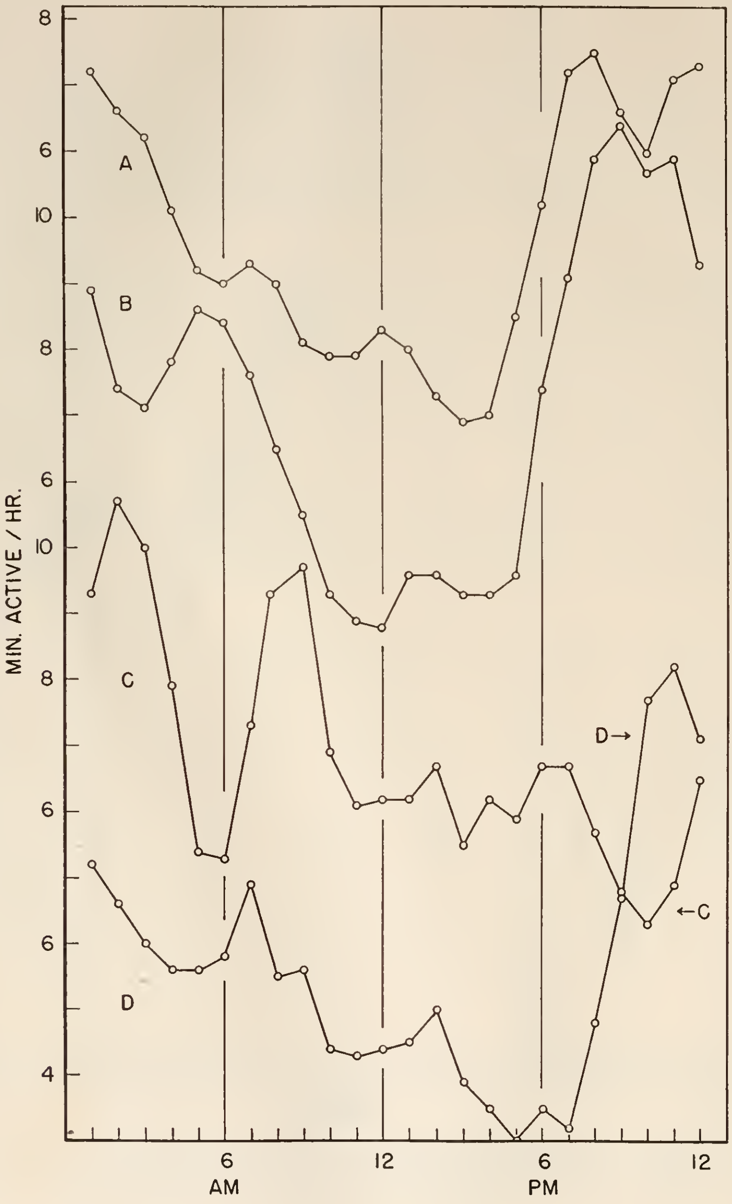

FIGURE 3. The average hourly activity of seven salamanders. A. May 14–20 (3rd quarter-New Moon). B. May 21–27 (New Moon-1st quarter). C. May 28-June 4 (1st quarter-Full Moon). D. June 5–11 (Full Moon-3rd quarter).

a low at 6 A.M. This was largely caused by a low average value of 2.9 at 6 A.M. Consequently, the sliding averages in the region around 6 A.M. are affected. Its significance is unknown.

Finally, the hourly averages for the last quarter of the lunar month are shown in Figure 3, D. Once again, depression appears evident, this time between 1 and 6 A.M., the range over which zenith occurred during the final seven days of this study.

The third method for showing the presence of a lunar rhythm is that which has been employed with much success by Brown (Brown, Bennett and Webb, 1954). For the purposes of this analytical technique, one may visualize the times of lunar zenith, and other corresponding lunar positions, as diagonals running downward and from left to right across the daily rows of data in Table I. As pointed out earlier, the time at which lunar zenith occurs for any one location is later with each successive day by about 50 minutes. Thus, the lunar day is of about 24.8 hours duration. A given lunar position completes one diagonal crossing of all the 24-hour vertical columns in about 29 days.[4]

Any lunar-associated influence may be made apparent if the corresponding lunar positions are aligned in vertical columns. Such a manipulation will serve also to "neutralize" the diurnal rhythm. To accomplish this, the day-by-day data of Table I were shifted to the left an average of about 50 minutes with respect to the preceding day. That is, day 1 was left in its normal hourly relationship, as was day 2, also. Then days 3 to 7 were each moved one hour to the left with respect to the clock hours of the preceding days. The data of day 8 were kept synchronized with those of day 7, but days 9 to 13 were each shifted one hour further to the left, and so on throughout the 29 days of data. Twenty-four vertical columns were retained by transposing, in sequence, the data which extended to the left beyond the first value of day 1 to the right side of the table.

Figure 1, C, shows the results of this analysis. Since zenith occurred at approximately 6 A.M. on the initial day of this experiment and all zeniths of the succeeding days were aligned with it, the position of zenith is indicated in the sixth column in Figure 1, C. This analysis provides further evidence that a depressive effect, which modulates the diurnal rhythm, is associated with the time of lunar zenith.

DISCUSSION

Upon comparison, it will be observed that the amplitude of the average cycle represented in Figure 1, A is about twice that shown by Figure 1, B. This difference may be explained upon the basis of two possible reasons, both of which may apply. When the animals are in alternating light and darkness (Fig. 1, A), the persistent rhythm may be amplified due to a direct influence of light intensity on locomotion. In continuous darkness the rhythm may fail to attain fullest expression, being "damped," but maintaining essentially the same frequency.

A second contributing cause for the difference in amplitude, and one which, in addition, may explain the occurrence of the minor minimum around midnight in Figure 1, A, could be that, in the period represented, only the first half of the A.M. hours, approximately, were subjected to the postulated depressive lunar influence, whereas all hours of the period represented by Figure 1, B were exposed to this influence. Thus, Figure 1, B presents the average diurnal rhythm with the lunar influence "neutralized," but nevertheless with lowered amplitude caused by the lunar depression. Figure 1, A is possibly distorted, in part, by the lunar influence, but most of its values were little affected by lunar depression.

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[4] The synodical lunar month, the period from one new moon to the next, has a mean length of 29 days, 12 hours, 44 minutes, and 2.8 seconds.

The rhythm of locomotor activity, as determined in this experiment under constant laboratory conditions, is undoubtedly similar to the variations in activity of this salamander in nature. Park, Lockett and Myers (1931) note that in the forest this salamander apparently passes the day beneath logs and stones and becomes active by 8:45 P.M.

The possession of a rhythm that assists in regulating the activity of the salamander, as described here, may be of supreme importance for the survival of the animal. As we have seen, it appears to have an activity rhythm that is determined by two components: (1) the diurnal activity pattern, which tends to keep activity minimal during the daylight hours and maximal during the night hours, and (2) the lunar modulation, which alters the diurnal pattern so as to minimize activity on moonlit nights. Thus, the inference is that the animal forays out of its hiding niche on nights when there is little or no moonlight and, hence, when there is less exposure to predators.

Since the period of study extended through only one lunar cycle, it cannot be unequivocably stated that the lunar-frequency modulation constitutes a lunar rhythm, but due to its close correlation with the lunar cycle and the similarity of this modulation with lunar rhythms that have previously been described (*cf.* Brown, Freeland and Ralph, 1955), it appears very likely that it is a lunar rhythm.

Though there appears to be a causal relationship between the lunar cycle and the lunar modification, there need be no direct inductive influence of the moon affecting the organisms. Diurnal rhythms show a causal relationship to the day-night cycle, but, as several studies have shown, the phase relationships of rhythms to the day-night cycle need not be fixed. Thus, the apparent "influence" of the moon on the activity rhythm may be only a persisting behavioral pattern that continues after the inductive influence of the moon is removed. Just as the 24-hour solar cycle may be impressed upon the activity pattern, likewise the 24.8-hour lunar cycle may also be impressed upon the pattern. The two frequencies together would appear largely to determine the overt expression of activity.

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SUMMARY

1. The salamander, *Plethodon cinereus,* shows a diurnal rhythm of locomotor activity, both in alternating 12-hour periods of light and darkness, and in continuous darkness.

2. The diurnal rhythm is strongly modified by a depressive influence that is apparently associated with the time of lunar zenith.

3. The activity of the animal at any given time is a function of the diurnal and lunar influences operative at that time.

4. The significance of the rhythm to the animal in nature is discussed.

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STUDIES ON THE EFFECTS OF IRRADIATION OF CELLULAR PARTICULATES.[1] II. THE EFFECT OF GAMMA RADIATION ON OXYGEN UPTAKE AND PHOSPHORYLATION

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It is now well established that dilute solutions of many enzymes are readily inactivated by ionizing radiation and that the presence of solute molecules, other than enzyme, decreases the effect of the radiation (Barron, 1954). Since the classical experiments of Dale (1940, 1942), it has been possible to explain the effects of low dosages of radiation on the basis of interactions between the protein molecule and the ionization products of water. These findings have led to the discovery of important facts about radiation damage and about the nature of enzymes. However, an important question is raised by this work; that is, are we able to draw valid conclusions about the biological effects of radiation from such studies? It is evident that cellular enzymes are not in a pure state, nor are they as dilute as is necessary to achieve effects in some cases. This makes it necessary to investigate the effects of radiation on enzymes under conditions which approximate those of the cell.

There are two obvious ways to do this. The most usual method is to radiate a whole organism (or cell) and then determine the enzymatic activity after radiation. The results of such work indicate that the damage to enzymes by lethal doses of radiation may be negligible (LeMay, 1951). However, there are a number of obvious difficulties in such work; and since the organisms do die eventually and do show loss of respiration in some cases (Barron, 1954), one is left with an unsatisfied feeling. For this reason, it seemed advisable to study the effects of ionizing radiation on cellular particulates (Yost, Robson and Spiegelman, 1956). The particulates of intermediate size (mitochondria) offer several interesting possibilities for such investigations: they can be isolated from the cell in good condition; they contain a large number of vital enzymes; they have a definite structure to which some of the enzymes are attached; the enzyme studied would always be in an environment similar to, if not exactly the same as, that in which it finds itself within the cell; and most important, the particulate is sufficiently large that one might assume that a major part of the damage done to enzymes within the particulate would result from the passage of the ionizing "particle" through the mitochondrion itself. This would mean that the effects of the radiation on the enzyme studied would be the same whether the mitochondrion was extracted or within the cell.

The experiments reported in this paper had a two-fold purpose: the establishment of a dose-inactivation curve for an enzyme known to be closely associated with the structure of the particulate, and the determination of the effect of ionizing radiation on the phosphorylation mechanism. Cytochrome oxidase was chosen as the test enzyme since it is known to be closely bound to the particulate and therefore might be an indicator of effects of the radiation on the particulate as a whole, and since it is of vital importance to the electron transport system. Furthermore, the implication of the cytochrome system in the production of mutations by ionizing radiation (Haas *et al.*, 1954) suggests this enzyme as an excellent starting point for the investigation of the effects of radiations on cells.

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[1] This work was supported by a grant, No. C–2154, from the National Institutes of Health.

Materials and Methods

White laboratory rats were starved overnight and sacrificed by a blow on the head. The liver was removed and placed in cold 0.85% KCl, where much of the blood was washed free. The liver was weighed and pressed through a bronze screen to remove connective tissue. The resulting mash was then suspended in 50 ml. of cold 8.5% sucrose containing 0.005 *M* disodium versenate and homogenized in a glass homogenizer with a "Teflon" pestle driven by a cone-drive stirring motor. The mitochondria were then separated from the rest of the homogenate by the method of differential centrifugation (Schneider, 1948). The mitochondrial fraction alone was kept.

In the studies of the effect of gamma radiation on the activity of cytochrome oxidase, the mitochondria were suspended in 2.5 ml. of sucrose-versenate per gram of original liver. For irradiation, a sample of the suspension was diluted 1 in 20 with distilled water. Five milliliters of the dilute preparation were put in a glass cup and irradiated in the beam of a 440-curie Co^{60} source. The radiation was filtered by a half-inch of lucite to remove beta radiation; the intensity of the radiation was 1000 r per minute. Controls were kept in a sheltered alcove outside the radiation room under the same conditions as the radiated material. The Warburg assays were run with the diluted preparations.

Treatment of the preparation for the determination of the effect of gamma radiation on phosphorylation differed from the above in some respects. The initial preparation was made by suspending the mitochondria in one ml. of sucrose-versenate per gram of liver. This suspension was then diluted 1 in 20 and radiated in a 25-ml. "Lusteroid" centrifuge tube. The controls were treated in the same manner, with the exception of the exposure to the radiation. After the radiation, control and treated suspensions were centrifuged, and the mitochondria re-suspended in one ml. This final suspension was assayed for phosphorylation.

The cytochrome oxidase activity was estimated manometrically by the method of Hogeboom, Claude and Hotchkiss (1946). The main compartment of each vessel contained: 0.35 ml. of mitochondrial suspension, 0.1 ml. Sorenson phosphate buffer (pH 7.4), 1 ml 1.3×10^{-4} *M* cytochrome-*c* (Sigma, horse-heart) in 0.85% NaCl, and 0.15 ml. 0.005 *M* $AlCl_3$. The center well contained 0.1 ml. 5 N KOH, and the side arm held the reducing agent, 0.15 ml. 0.228 *M* sodium ascorbate.

Estimation of phosphorylation was concluded by a modification of the method of Maley and Lardy (1954), using succinate as the substrate. The main compartment of the vessel contained: 0.3 ml. (30 μM) phosphate buffer (pH 7.4), 0.3 ml. 0.1 *M* sodium succinate, 0.8 ml. 8.5% sucrose, 0.1 ml. (0.3 μM) cytochrome-*c*, 0.3 ml. (6 μM) ATP (Schwartz, neutral), 0.1 ml. (30 μM) $MgSO_4$, 0.1 ml. (40 μM) KF, and 0.5 ml. of the mitochondrial suspension. The center well contained

0.1 ml. 5 N KOH, and the side arm held 0.5 ml. (20 mg.) of hexokinase (Pabst). To assure that the final pH of the reaction would be 7.0 or higher, the pH of some of the more acid reactants was adjusted with NaOH before addition to the flasks. Failure to do this results in lowered oxygen uptake and lowered phosphorylation. Readings of the oxygen uptake were taken for 30 minutes, after which time the reactions were stopped with TCA and the phosphate determined by the Lowry-Lopez method as presented by Glick (1949).

Assays of oxidase activity were made at 38° C.; assays of phosphorylation were made at 25° C. Assays of oxygen uptake were made in triplicate; assays of phosphorylation were made in duplicate. All experiments were repeated at least three times.

RESULTS

Table I presents the data obtained from radiation of mitochondrial preparations of differing age. The preparation labeled "Day 1" was radiated on the same day that it was extracted; the preparation labeled "Day 2" was radiated on the following day; etc. These data show that cytochrome oxidase is extremely resistant to gamma radiation. This is in accord with the earlier studies of Barron *et al.* (1949). Nevertheless, although the data are extremely erratic below 10,000 r, some effect is achieved with doses as low as 2500 r. There is little indication that a maximum has been reached at 40,000 r. It is necessary to comment on the variability shown by the data. The figures are averages of several runs done with different rats. We have found that preparations from different rats give different results. In the case of the data obtained with 4000 r, Day 2, the inactivation varied from 12.1 per cent to 36.5 per cent. Whether this intrinsic variability is the result of differences in age, sex, or physiological condition, we are unable to judge at this time. In addition, there is always the problem of variation in the concentration of the preparations. All dilutions are made from suspensions made up as 2.5 ml. per gram of liver extracted. There is no reason to suppose that the number of mitochondria will be the same in each case. Until studies are done in

TABLE I

Inactivation of cytochrome oxidase by gamma radiation

Dose *r*	Day 1		Day 2		Day 3	
	No. runs	Per cent inactivation	No. runs	Per cent inactivation	No. runs	Per cent inactivation
2,500	2	1.8±4.1	6	6.4±3.8	6	5.7±6.0
4,000	8	4.8±2.4	18	18.9±2.9	4	8.7±2.2
5,000	13	9.4±2.9	18	8.8±2.1	12	10.2±4.4
10,000	6	10.5±2.2	10	6.5±1.8	9	10.5±2.6
12,500	8	16.7±2.4	8	25.7±4.0	10	27.1±3.2
15,000	14	17.4±2.9	17	26.7±2.0	7	26.5±1.6
20,000	19	29.9±2.4	16	29.7±2.3	16	26.7±2.1
30,000	5	41.3±3.5	11	34.6±2.5	6	31.0±6.5
40,000	6	49.1±2.8	10	42.8±4.5	12	34.1±3.3

TABLE II

The effect of aging on the inactivation of cytochrome oxidase

Dose *r*	Morning		Afternoon	
	No. runs	Per cent inactivation	No. runs	Per cent inactivation
5,000	4	0.0±2.0	4	16.9±2.0
15,000	4	7.4±1.5	4	21.9±2.4
20,000	4	24.5±4.5	4	39.6±1.8

which the number of mitochondria in each sample are the same, no conclusions can be drawn about the variability between rats.

The data in Table I indicate that the preparations become more sensitive to low doses of radiation with time. On the first day, a dose of 2500 r produces little or no inactivation; on the second day, it produces about 6 per cent. This is shown in a much more striking manner in Table II. In this case the data from some runs done on the first day are broken down into those done in the morning and those done in the afternoon. In all cases, a preparation was radiated in the morning and afternoon, the only difference being the age. It is evident that when the mitochondria are first extracted they are much more resistant to radiation, particularly at the lower doses. At high doses, Table I indicates that there is a progressive decrease in sensitivity with age. Intermediate doses are erratic. As a result of

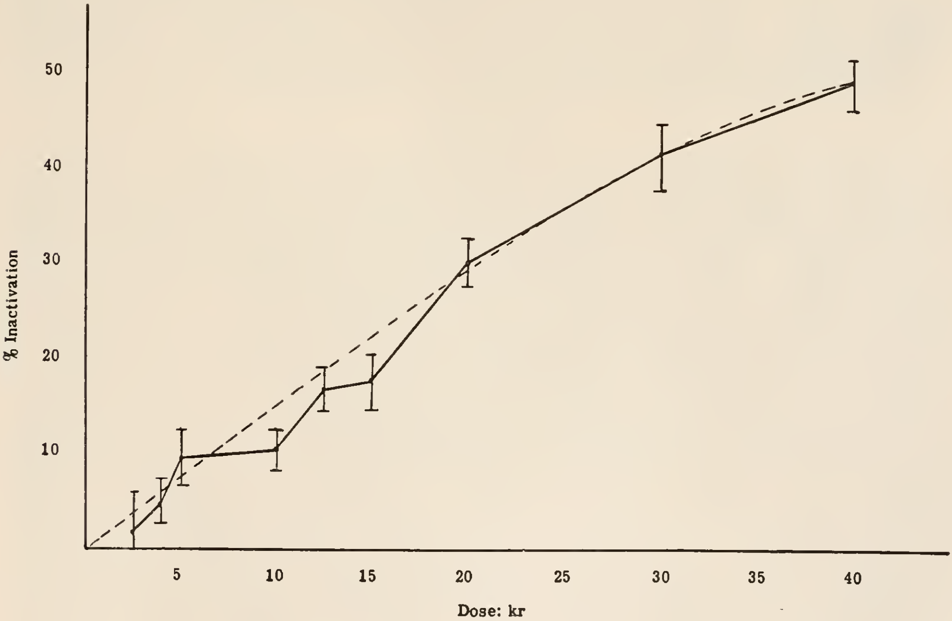

FIGURE 1. Inactivation of cytochrome oxidase by gamma radiation. Points represent means, with standard errors shown as limits. Preparation radiated the same day it was extracted. The dotted line represents the best fit of a regular curve.

this aging effect, the inactivation curve shown in Figure 1 was constructed on the basis of the first-day results only.

The data presented in Table III show that the oxidative phosphorylation mechanism is far more sensitive to radiation than cytochrome oxidase. All runs were done with a fresh preparation. There is no appreciable difference in the age of the preparations. In this case, each run represents a different rat, so that the variability results from this alone. It can be seen that there is little effect of the radiation upon the oxygen uptake in any case. The stimulation of phosphorylation by 2500 r is slightly greater than would be expected on the basis of increased oxygen uptake, when calculations are made using a theoretical P:O ratio of 2 for succinate.

Table III

Inactivation of phosphorylation by gamma radiation

Dose	Phosphate uptake:		Per cent decrease	No. treated	O_2 uptake, % decrease
	Controls	Treated			
2,500	6.6±1.0	6.8±0.64	−3.0	10	−9.3
5,000	8.0±1.3	7.5±1.1	6.2	10	7.9
10,000	6.7±1.1	5.1±0.62	23.8	10	6.5
15,000	9.1±1.4	6.4±0.47	29.6	11	5.3
20,000	9.2±2.5	4.4±1.3	52.2	3	7.5
30,000	11.8±2.6	4.9±1.0	58.5	5	5.4
40,000	6.2±0.93	1.7±0.36	72.6	6	8.3

In all tables the data are shown as means ± standard error.

Discussion

The data presented in this paper indicate that the inactivation of cytochrome oxidase by gamma radiation follows an irregular course. In all cases, there is a plateau reached at 5000 r to 10,000 r, followed by a sharp increase between 10,000 r and 12,500 r. The plateau is not lost upon aging (if anything, it is intensified); nor is it absent in fresh preparations. Table II indicates that in very fresh preparations there is a sharp increase between 15,000 and 20,000 r. It appears that the age of the preparation merely increases the dose necessary to cause the "jump." This observation requires special consideration. In the oxidation of cytochrome-*c* by ionizing radiation, it has been shown that the effects of radiation doses below 10,000 r are completely reversible (Barron, 1954). It is possible that part of the curve (Fig. 1) between 10,000 r and 15,000 r represents a shift from indirect to direct effects upon the particulate. Since we are radiating the entire particulate system, we can expect that the terminal oxidase of this system is constantly being reduced by substrate (either the supplied cytochrome-*c* or internal metabolites). This continual reduction may protect the cytochrome oxidase from the effects of the radiation, either during the time of radiation, when it must draw upon its internal supplies, or during the assay procedure, when it is supplied with a reducing agent which may effect post-irradiation recovery. Support for this idea comes from the aging effect shown in Table II. If the oxidase were being protected by materials within the mitochondrion at the time of radiation, we would expect that aging the preparation would lead to depletion of the internal stores with a consequent lowering of the protective effect. This is what is observed. At higher doses this protective effect is apparently negligible compared to the dose administered. Thus the effect of the radiation is reduced with age as might be expected from the protective effect of particulates whose oxidase has been inactivated by causes other than radiation.

It may be argued that the protection is merely the result of the various solute molecules which are not removed by washing during the extraction. It is difficult to decide whether there is a specific type of protection resulting from the generation of "reducing power" from the substrate, or whether there is a non-specific protective effect of additional substrate (Dale, 1942). It is clear that the effective substance is lost during aging; therefore, it seems sure that the protective substance must be within the particulate to be effective. If the substance were merely lost from the mitochondrion, it should be in the suspending medium which is diluted before radiation. The same concentration of substance would be present in either case. Such considerations raise the question of whether the effects of the radiation are independent of the concentration of the mitochondria. It might be suspected that only those ionizations produced within a mitochondrion would affect the enzymes within the structure. The particulate is sufficiently large to justify such an assumption. However, this does not prove to be the case. It was first established that preparations diluted in sucrose were less easily inactivated than those diluted in distilled water. This suggests that the ionization products of water are acting on the mitochondrion and that the sucrose is acting as a non-specific protective agent. Unfortunately this is not a clear test. The sucrose might be causing a change in the osmotic condition of the particulate, so that the protective substance (normally lost by aging) is more concentrated within the mitochondrion, or is not lost as readily. However, the data presented in Table III indicate that there is a dependance upon dilution. It can be seen that in a preparation 2.5 times as concentrated as that used to obtain the data in Table I, 40,000 r produce 8.3 per cent inactivation of oxygen uptake, whereas they produce 49.1 per cent in the dilute preparation. Clearly, 49.1 per cent is much greater than 2.5 times 8.3 per cent. Studies of intermediate dilutions bear this out. It is necessary to conclude that the effect of radiation upon the particulates is indirect in the sense that any solute molecule outside the mitochondrion will exert some protective effect upon the enzymes which are internal. Therefore, we must further conclude that the effect of the protective substance which is lost upon aging must be the result of some action it has *within* the mitochondrion, and that the loss which occurs with age probably results from the destruction of the substance by the particulates. As pointed out above, it is impossible to be sure of the mode of action of this protective agent, but the generation of materials which keep the enzymes in a reduced condition seems a likely mechanism.

A second explanation of the sudden "jump" in inactivation is that at doses over 10,000 r the particulate structure is undergoing severe change. This might result in the freeing of enzyme molecules, with consequent dilution of protective substances or in the loss of function of one part of the system with its release from another part. This seems a very unlikely mechanism. The fact that cytochrome oxidase can be freed from the rest of the particulate and still retain its activity

(Eichel *et al.* 1950) makes such an hypothesis difficult to maintain. Examination of the preparations by phase contrast did not show any radical changes; however, any alterations of ultrastructure could only be detected by other methods.

The dilution effect deserves additional comment. These experiments were originally designed in the hope that we would be able to approximate the biological condition. Radiation studies with dilute solutions of pure enzymes are informative with regard to radiation problems, but somewhat off the point for biological problems. No cellular system exists which is a single molecular species. Attempts to show that enzyme damage is the cause of radiation death in cells have been relatively unsuccessful (LeMay, 1951). On the other hand, the cell is so complex that it may die from many different causes, and it is difficult to be sure that one is investigating the right system in any particular case. It seemed necessary to investigate the problem in a system which had biological characteristics but which was not quite so diffuse as a whole cell. The particulates appear to offer such a system. It is possible to extract a "package" of enzymes, each of which has a relationship to other enzymes in the "package." The "package" resembles a cell in many aspects, but it is much simpler in its total organization. Furthermore it seemed that it was of sufficient size that only those ionizations produced within the mitochondrion would have any effect on the internal enzymes. This would be of great importance to the radiobiologist since it would indicate that there are sub-cellular bodies which can be considered to be separate from the rest of the cell, with regard to radiation damage. The chromosomes are frequently considered to be bodies of this type. Unfortunately this does not seem to be the case. The data presented in this paper indicate that ionizations external to the mitochondrion may cause internal damage, in a system containing only particulates and distilled water. It would appear that, at the doses studied, the ionization products of water are capable of producing their effects over relatively great distances, or that the effects of these products on the surface of the mitochondrion are capable of reducing the activity of the enzymes which are internal. This is of special interest as it has been suggested that the major effect of radiation on cytochrome-*c* is produced by hydroxyl radicals alone (Barron, 1954). Knowledge of the exact position of the oxidase in the particulate would be necessary to any final conclusion about these effects. However, the difficulties encountered in the extraction of cytochrome oxidase (Eichel *et al.,* 1950) suggest that the enzyme is internally bound.

From the foregoing discussion, it seems evident that we cannot conclude that cytochrome oxidase is damaged to any great extent by radiation doses used in most biological studies. It is difficult to know the concentration of the particulates in any cell, but it seems that the final suspension used in the phosphorylation studies (one ml. per one gm. liver) would best approximate the natural condition in liver cells. At no time was radiation given to particulates at this dilution. The strongest preparation ever used (during radiation) was 20 times diluted. At this dilution (Table III) there is little inactivation at 40,000 r. It is interesting that the effect on the oxidase seems to be the same for a wide range of doses. It is possible that in any dilution some small fraction of the activity would be lost, but this seems to be an unlikely cause of cell death. It is necessary to note that many cells do not have the high concentration of particulates which liver has. In these cases the effective doses necessary to inactivate cytochrome oxidase might fall within the limits of biological experimentation, even with the protective substance of the cytoplasm present. In highly organized forms, the failure of one part may result in the death of the whole, so that damage to this system might *in some cases* result in death. This is particularly true of the forms which require fantastic doses of radiation to induce lethal changes.

When one considers the data in Table III, it becomes apparent that the oxygen uptake may be a faulty criterion for estimate of the health of a cell. These data show quite clearly that the phosphorylation mechanism is much more sensitive than the oxidase. Here we have a case in which over 70% of the ability to conserve energy as organic phosphate is gone with no apparent effect on the oxygen uptake. Considering the vital role of this system in the life of the cell, it seems quite probable that disruption of vital processes would result from the loss of 25 per cent (or less) of the ability to phosphorylate. We do not know how prevalent this loss is in the whole system. In these studies only the uptake which resulted from the oxidation of succinic acid was measured. It is possible that the whole phosphorylation mechanism of the particulates is damaged. Studies to determine the extent of the damage and to determine whether phosphorylation is carried out by a single system for all substrates are now in progress. In any case, it is evident that the phosphorylation mechanism is subject to destruction by ionizing radiation and that inactivation is achieved in relatively concentrated preparations which are very fresh. Although such preparations must be high in the concentration of the protective substance found for cytochrome oxidase, there seems to be little protection of the phosphorylation mechanism. This may be an indication that the protection mechanism is specific for the electron transport system (if not for cytochrome oxidase itself) or that the phosphorylation mechanism is extremely sensitive to radiation.

These data suggest an explanation for several different phenomena which have radiation as their sole common element. The phenomena are: induced crossing-over, induced tumor formation, and the general protective effect exerted by reducing compounds of the cysteine type. It has been suggested that one basis for the changes in genetic crossing-over induced by radiation is the alteration in the availability of phosphate linkages within the chromosome (Yost and Benneyan, 1957). It is evident that alterations in the phosphate pool of the cell must result from the type of damage described in this paper. Indeed, it is to be expected that such changes will have drastic effects on the chromosome structure, as many studies have already indicated (Haas *et al.,* 1954). It is also possible that *some* radiation-induced tumors are the result of the uncoupling of the oxidative metabolism of the cell. This could result in a situation similar to that which Warburg has suggested several times (Warburg, 1956). In cases in which oxygen uptake alone is measured, there is no assurance that the phosphorylation mechanism is functioning. Lastly, the general effect of reducing agents may be more than the maintenance of vital sulfhydryl groups. It is possible that the actual utilization of these compounds as reducing agents in the general metabolism will result in the protection of many non-sulfhydryl systems of the cell.

SUMMARY

Data are presented which indicate that the phosphorylation mechanism is much more sensitive to gamma radiation than cytochrome oxidase. It is suggested that

the utilization of substrates by enzymes within the particulate may protect these enzymes against ionizing radiation. Various consequences of these findings are discussed.

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THE GENERAL FORM OF EXCRETION IN THE LOBSTER, HOMARUS[1]

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While there exists an extensive literature on the regulation of inorganic ions in the higher Crustacea (Krogh, 1939; Robertson, 1949, 1953; Prosser *et al.,* 1950), the experimental study of nephridial function has received little attention, due apparently to a lack of a method for securing repeated samples of urine. This report gives such a method and it is an attempt to develop an integrated picture of the regulation of the internal environment of a single species, *Homarus americanus* (a lobster), through the experimental study of a variety of organic and inorganic substances.

Comprehensive inorganic analyses of lobster blood have been made by Cole, and Smith in Cole (1940), and of blood and urine by Robertson (1939, 1949, 1953), and by Robertson and Webb (1939). The nephridial anatomy has been described by Marchal (1892), Waite (1899), and Peters (1935). Histologically there is no structure corresponding to the vertebrate glomerulus. The study by Cuénot (1895) of the differential concentration of dyes by various organs in different decapods is of considerable interest.

MATERIALS AND METHODS

Because of the large number of substances studied and of experiments performed, individual techniques will be described in context. Over two hundred lobsters were studied. These were largely from the Mt. Desert Island region, but a few were from Nova Scotia. For all experiments the animals were given at least twenty-four hours to equilibrate to the sea water at the Laboratory.

Urine from the nephridial bladders was secured at will without catheters by a technique shown to us by Dr. P. R. Wilder of the Atlantic Biological Laboratory, St. Andrews, N. B. If one's thumb is placed between the bases of the pereiopods with the fingers over the carapace, preferably with the tail flexed, squeezing the hand results in two jets of urine from the nephridiopores which can be collected in test tubes held by the other hand. This technique works best with so-called "hard-shelled" lobsters. The value of the technique was tested critically with twelve lobsters. After expressing the urine, the carapace was cut open and the bladders were examined directly. In eight animals the bladders were empty; the highest residuum

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[1] Aided by a grant from the New York Heart Association.

of the remaining four was 10% of the expressed volume. Thus the technique, while not perfect, is serviceable. Since not all lobsters from commercial pounds form urine, and since individual animals differ in the ease with which urine can be expressed, another critical study of twelve lobsters was made which showed we could differentiate accurately the anuric specimens. As with many other techniques, judgments depend more on practice than on formal rules. In general, however, if the animal has good muscle tone, if the opercular flaps of the nephridiopores are elevated on squeezing, and if the lobster is fresh from sea water, the animal is producing urine. It is necessary sometimes to flick the opercular flaps with the fingernail to start the flow of urine.

Safe occlusion of the nephridiopores can be made by placing a wide rubber band across the pores, crossing the band over the dorsal carapace, and then securing the band on the posterio-ventral margin of the gill covers.

Test substances can be injected into the hemocoele, or more safely can be pipetted through the mouth into the stomach from which they are absorbed. Blood is most easily withdrawn from the ventral surface of the abdomen. The only reliable method we found for the prevention of clotting was to whip or shake the blood, filter it several times (Cole and Kazalski, 1939), and then preferably dilute it.

RESULTS

The data [2] are presented in three sections: Organic Substances, Inorganic Substances,´Intake and Output, with the discussion pertinent to each *in situ*.

Organic substances

1. *Inulin.* Single injections into eight lobsters of inulin were followed up to 28 hours with up to 5 sampling periods. The analytic method of Schreiner (1950) was used. For blood levels which ranged between 17 and 1.4 mg. %, the urine-plasma (U/P) ratios were essentially one (1.0–1.1), with the concentrations in the blood and urine falling with the same slope. Forster and Zia-Wohlrath (1941), working with higher blood inulin levels (192–65 mg. %), also found inulin U/P ratios of one. In the lobster, inulin is not secreted by the nephridium as reported for the crayfish (Maluf, 1941). Since the inulin U/P ratio is substantially one, the U/P ratios alone of other test substances should offer a reasonably accurate guide for determining the partitioning ability of the nephridium, and there is no need to compare the concentration of a test substance with the urinary concentration of inulin in subsequent experiments, as would be necessary if there were a differential separation of water and inulin.

2. *Vertebrate hemoglobin and plasma proteins.* To test the permeability of the nephridium to large molecules, solutions of hemoglobin prepared from hemolyzed red cells of the dogfish (*Squalus acanthias*) were injected into the hemocoele of eight lobsters. The normally clear urine promptly became pink and remained so for several days. Plasma proteins from the dogfish were injected into four lobsters. On gentle evaporation, the urine jelled, an unnatural event. While it was not de-

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[2] The following technical assistance is acknowledged gratefully: Dr. E. L. Becker, freezing point depression; Dr. Klaus Brunn and Xenia Boysen, urea; Dr. Roy P. Forster, inulin and PAH; Drs. Henry Heinemann and Wilbur Sawyer, chloride; Drs. Martin Rubin and Frederick Berglund, magnesium and calcium; Dr. Charles G. Zubrod, glucose.

termined that the original molecules were recovered in the urine in their original state, it does appear that the nephridium of the lobster is permeable to molecules of the size found in the blood of fish. The natural urine of the lobster is protein-free (Forster and Zia-Wohlrath, 1941). Urine treated with the standard protein-precipitating agents shows no increased Tyndall effect or clouding.

3. *Glucose.* The Hagedorn-Jensen sugar titration method (Peters and Van Slyke, 1946) gave in mg. % urine/blood values of: 0/22; 0/24; 0/27; 0/28; 0/32; 0/37; 0/39; 0/40. Two urine values were slightly positive: 2/31; < 5/31. It is uncertain whether these are genuinely positive values since the method involves subtracting from the titrated value the value of a blank. Morgulis (1922) reported previously blood "glucose" levels of 19–26 mg. % for *Homarus,* with a considerable variability in other decapods (1922, 1923). That the test was measuring sugar and not some other reducing substance is indicated by the fact that in lobsters with the heart destroyed, the blood was free from reducing substance fifteen minutes later.

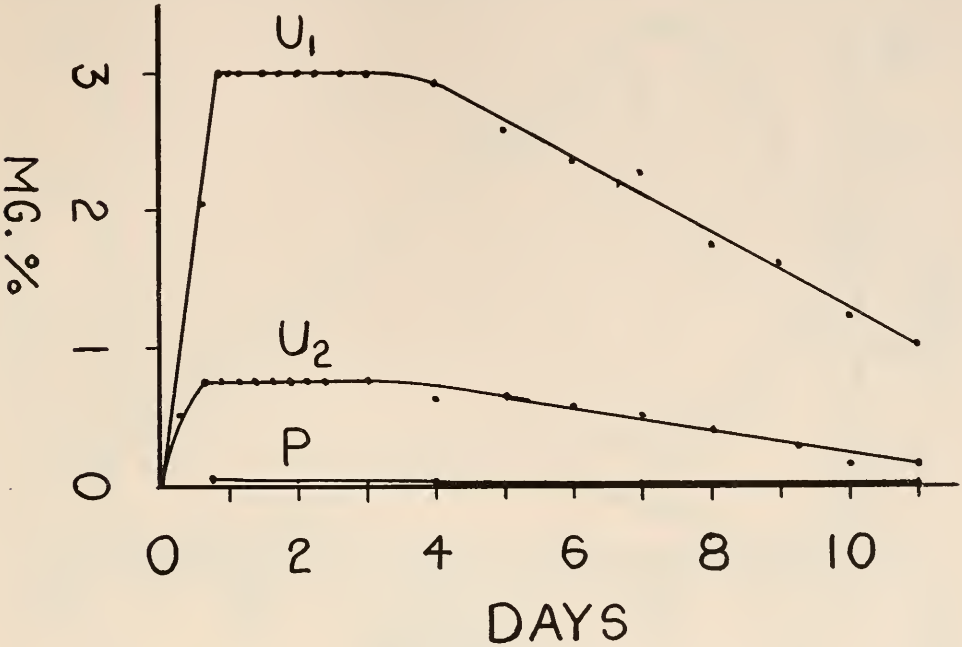

FIGURE 1. Concentrations of phenol red in urine (U_1, U_2) and blood (P) in two lobsters with 6 and 4 mg. of dye pipetted into the stomach. The blood levels were so similar that they are given as one curve, scarcely distinguishable from the horizontal axis. At these blood levels the dye is obviously secreted by the nephridia, but leaves the animal slowly for reasons explained in the text.

With the injection of exogenous glucose, the lobster's nephridium behaves like the vertebrate kidney. Up to blood levels of about 100 mg. % the urine remains free of glucose. Urine-plasma ratios were: 0/81; 0/84; 0/94; 0/106. With further elevation, glucose spilled into the urine, *e.g.,* 30/202; 30/210, and at blood concentrations of 400–500 mg. % the U/P ratio approached one. The report by Forster and Zia-Wohlrath (1941) of a glucose U/P ratio of one in *Homarus* is due undoubtedly to the high level of glucose employed.

Phlorizin resulted in glycosuria with or without priming by sub-liminal exogenous glucose (10 lobsters). In short, under normal conditions there seems to be an active mechanism which excludes glucose from the urine, a mechanism which can be poisoned by phlorizin.

4. *Phenol red.* Phenol red was extracted from the blood by acid alcohol, read colorimetrically at 440 mμ, alkalized and read again at 550 mμ. Urine and stomach fluid were treated similarly. It was found desirable to use control blanks from individual lobsters, and not to rely on a generalized control zero. Over thirty animals were studied.

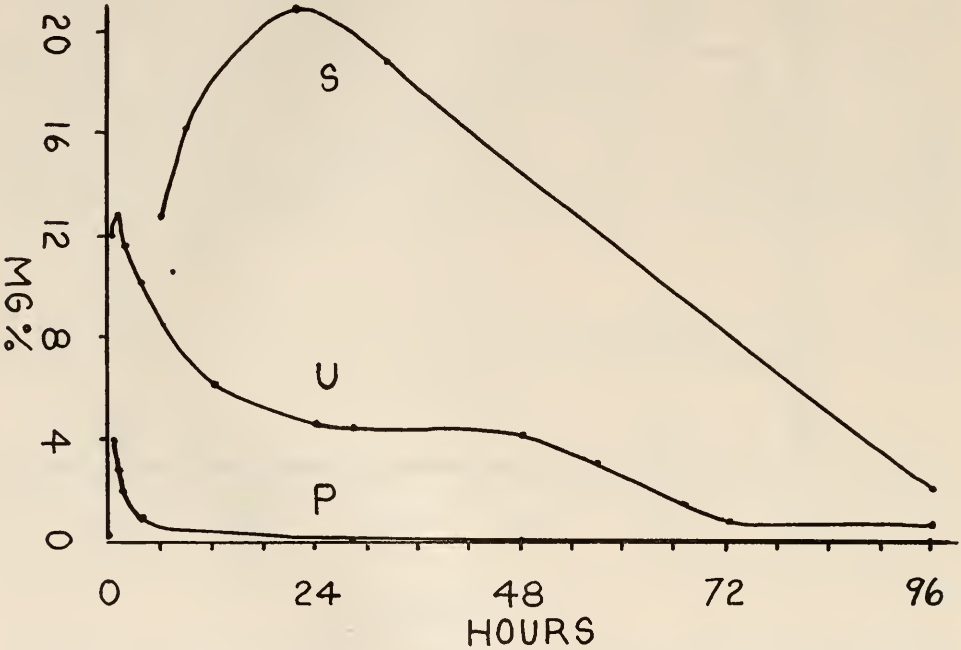

FIGURE 2. Urinary concentrations (U) of phenol red after injection into the hemocoele, over wider blood levels (P) than shown in Figure 1. Note the concentration of the dye in the digestive fluid (S). The isolated point is a urine value.

The pattern of nephridial excretion of phenol red is seen in Figures 1 and 2. At low plasma concentrations (Figs. 1, 2), the dye is clearly concentrated by the nephridium (the U/P ratio is a valid criterion; see paragraph on inulin). As the plasma level is raised, the U/P ratio approaches one (*cf.* Fig. 4). This is the pattern for all substances "secreted" by the nephridium; concentration is evident at low plasma concentrations but is apparently swamped by high plasma concentrations. It is to be remembered that the lobster's kidney is the elaboration of only one pair of nephrons.

Phenol red is not lost or absorbed through the gills or body covering. Four lobsters were injected with 5–10 mg. and were placed for 18 hours in volumes of water sufficiently small to detect a small fraction of the injected dye. No phenol red was detected in the external medium. Four animals were placed for 18 hours in sea water containing 300 mg. % phenol red. No dye was detectable in the blood, urine, or digestive gland. Phenol red placed in the stomach is absorbed. After injection of phenol red, this substance was detectable in the cells of the gills. The uptake of dye by branchial cells is seen more dramatically with Evans blue. After a single injection of Evans blue, the gills become a bright blue, and so remain for at least a month, when no dye is detectable in the blood. The dye obviously entered the cells of the gills, but did not move easily either out to the sea water or back to the blood. One can speculate that for certain substances passage through the branchial cells involves at least two steps: cellular uptake or penetration, and extrusion.

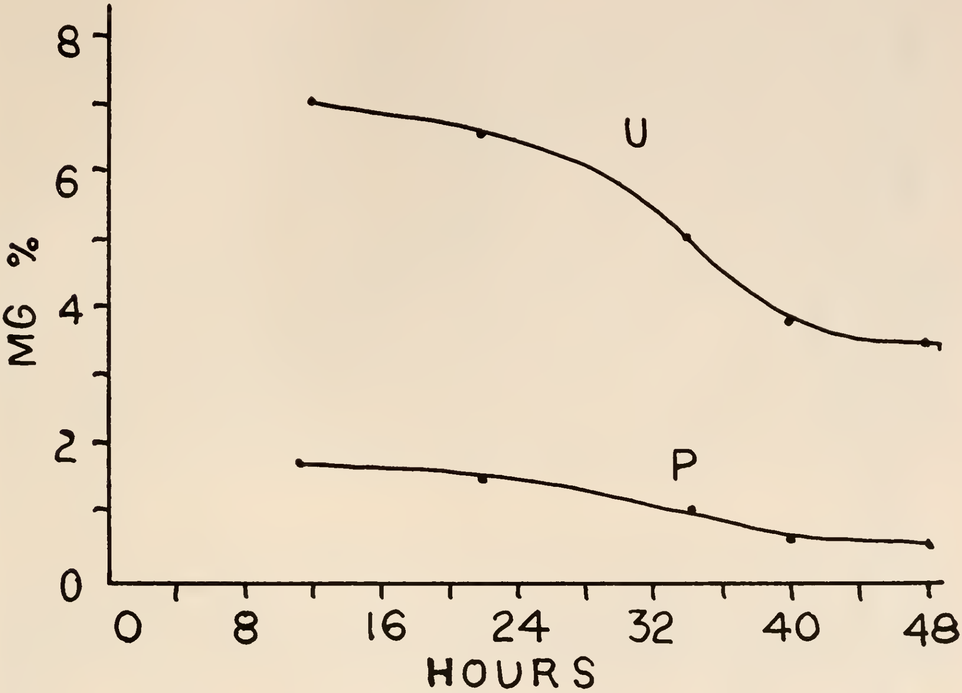

FIGURE 3. Concentrations of para-aminohippurate in urine (U) and blood (P) after a 4-mg. stomach infusion. Nephridial secretion is evident.

Fecal loss of phenol red is minute. The dye, however, is taken up by the digestive gland. Extracts from the gland show phenol red when after injection the blood level has subsided to insignificant amounts. The dye absorbed by the digestive gland is secreted with the digestive juice at concentrations greater than those of the urine (Fig. 2). Whether the individual cell of the digestive gland can concentrate more than the nephridial cell requires further study, since the mass of the digestive gland is greater than that of the kidney, and the movement of water through these two types of cells probably is not the same.

The dye secreted with the digestive juice is not lost with the feces or voided by mouth, but is reabsorbed, and cycles back and forth between the stomach and the digestive gland, *slowly* being lost with the urine (Fig. 1). The nephridia seem, therefore, the principal port of exit for phenol red.

5. *Sulfobromophthalein* (*bromsulfalein*). This dye, analyzed colorimetrically, was explored over blood concentration of 0.02–100 mg. % in eight lobsters. The U/P ratios were between one and two with no tendency to rise at the lower plasma concentrations. It is concluded that bromsulfalein is not concentrated by the nephridium. In the digestive juice, however, bromsulfalein is concentrated more strongly than phenol red. Without further quantitation, a numerical comparison is dangerous, but with comparable doses (10, 25 mg.) of dyes into lobsters of comparable weight, the digestive juice bearing bromsulfalein showed about four times the concentration of dye as that bearing phenol red. The differential ability of the hepato-pancreas to concentrate this dye like the vertebrate liver indicates that the name for this organ is more than an anatomical appellation. Like phenol red, the loss from the lobster of bromsulfalein is very slow.

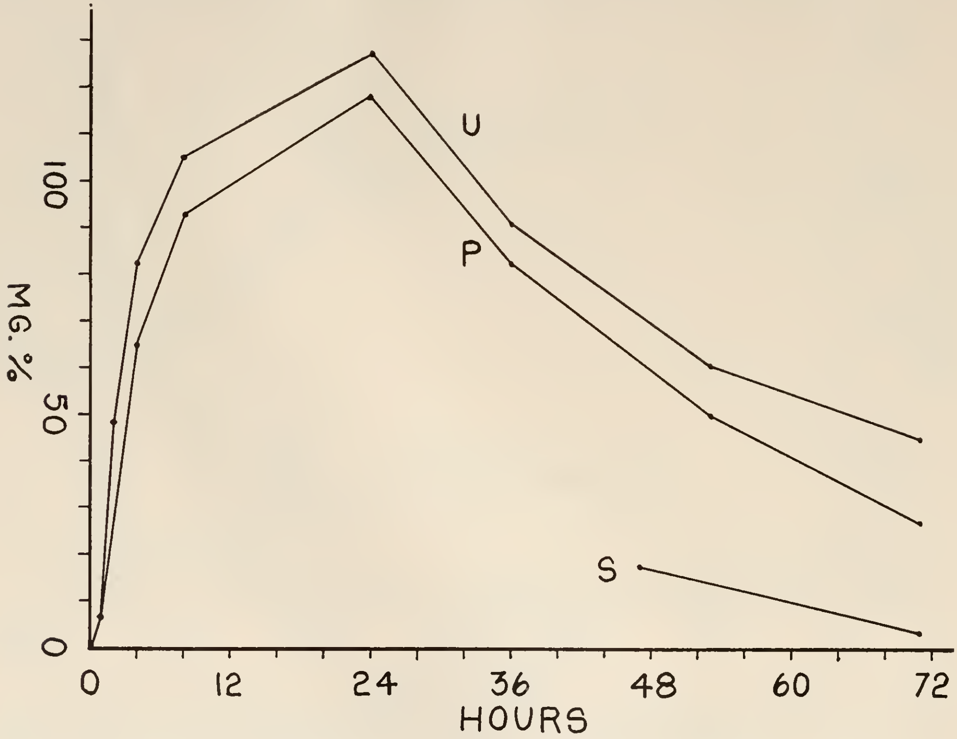

FIGURE 4. Urine (U) blood (P), and digestive juice (S) values following a 400-mg. dose of PAH, placed in the stomach. At high blood levels the U/P ratio approaches one. As the blood level falls, the U/P ratio increases. This sort of curve is characteristic for substances secreted by the nephridia.

6. *Para-aminohippurate* (PAH). Using the analytic method of Smith *et al.* (1945), nephridial concentrations were explored in eight lobsters with blood levels

of 120–0.5 mg. %. At low blood levels (Fig. 3), PAH is concentrated in the urine and the U/P ratio rises as the blood level falls. As the blood concentration is raised the U/P ratio approaches one (Fig. 4). The digestive gland does not concentrate PAH; rather, the stomach juice concentration is below the blood concentration.

7. *Urea and nitrogen.* Analyses for urea and volatile ammonia were made by the method of Seligson and Seligson (1951). For non-protein nitrogen (NPN) sulphuric acid digests were nesslerized directly. Urea and volatile ammonia were undetectable in blood and urine (10 animals). The natural range for NPN was 5–32 mg. %. Morgulis (1922) found a range of 12.5–13.3 mg. % for *Homarus,* with a wider range in other decapods. No constant relationship was observed between blood and urine NPN. The U/P ratios varied from 0.7–6.0 in ten animals.

Exogenous urea is lost rapidly from the blood through the gills. Four lobsters with occluded nephridiopores were injected with 500 mg. urea, then placed in measured volumes of sea water. After one hour sea water/blood concentrations in mg. % fell between 12.8/12.65 and 19.6/14.25. Since the external volume was greater than that of the animals, most of the urea was in the external medium. In four other animals injected with 500 mg. urea and with occluded nephridiopores, no urea was detectable in the blood after the animals were 24 hours in running sea water. Morgulis (1922) found a rapid disappearance of blood urea and ammonium sulphate without nephridial occlusion in *Panulirus.* While currently there is no evidence that urea is the main nitrogen excretory product, the above experiments indicate a high in-out permeability to urea by the gills, and that this loss can occur without nephridial participation. The presence of a substantial blood NPN must indicate a low branchial permeability to certain nitrogenous compounds.

Inorganic substances

Chemical analyses for sea water, blood, and urine are given in Table I. In the paragraphs following are data on individual ions. The purpose of this study is to understand the range of capacities for dealing with various ions and their ports of entrance and exit. Robertson's studies (1939, 1949, 1953; Robertson and Webb, 1939) have defined at a non-experimental level the natural partition ratios for *Homarus vulgaris.* Robertson (1949) has criticized correctly the presentation of blood values in millimols/liter, and has emphasized the need for a correction for the presence of blood proteins. In working up his ratios he seems to have used a single average value of 29 g./liter protein for *Homarus,* although for a variety of decapods he found values ranging from 29–80 g./liter. Allison and Cole (1940) got values of 17.1–31.2 g./liter of hemocyanin for Mt. Desert Island lobsters. Our data from over thirty Mt. Desert Island lobsters show a very high variability in what we are somewhat arbitrarily calling blood protein. Values ranged from 11–62 g./liter, with about 50 g./liter as a generalized usual figure. The above values were secured, following one of the methods of Robertson (1949), by drying weighed calibrated volumes of serum to constant weight at 100° C. From the residual weight was subtracted the weight of electrolytes (Na, K, Ca, Mg, Cl, PO_4, SO_4), an arbitrary value for the water of crystallization derived from dried sea water, known organic substances such as NPN and glucose. In the absence of any evidence in the literature of massive amounts of some unknown substance, and since known organic substances occur in fractions of a gram/liter, the values here presented while perhaps not entirely pure, do seem to offer a fair picture of the order of magnitude and of the natural range of blood proteins. It perhaps should be emphasized the values presented represented a selection of living lobsters some of which were lethargic and sub-standard. The range for so-called normal lobsters was 35–62 g./liter.

It is obvious that unless the non-electrolyte concentration of the blood is determined for each *animal,* molal expressions for electrolytes are not entirely precise.

TABLE I

Analyses of lobster blood, urine, and of sea water

	Sea water	Blood	Urine
pH*	(7.6–8.0) [10]	(7.45–7.6) [16]	(7.4–7.55) [26]
Dried solids,** g./liter	(35.5–35.9) [10]	(71.2–98.6→47) [22]	(32.7–35.9) [8]
Organic solids, g./liter	—	50(35–62→11) [22]	(<0.4) [8]
Water, g./liter of blood	—	950(935–959→976) [22]	—
Milliosmols	(915–920) [12]	(920–952) [20]	(918–950) [20]
Sodium, mM/liter	440(428–445) [15]	472(451–488) [20]	474(454–486) [22]
Potassium, mM/liter	(9–10) [10]	(6–11) [14]	(4–10) [16]
Magnesium, mM/liter	(50–52) [8]	6.8(5.4–8.6) [15]	11.4(7.2–17.6) [15]
Calcium, mM/liter	(9–10) [8]	15.6(13.1–18.6) [15]	12.7(5.6–16.3) [15]
Chloride, mM/liter	503(476–515) [27]	470(465–490) [27]	505(490–520) [27]
Phosphate, g./liter	—	0.016(0.008–0.018) [8]	0 [16]
"Glucose," g./liter	—	(0.22–0.40) [10]	(0–0?) [10]
Non-protein nitrogen, g./liter	—	(0.05–0.32) [10]	(0.05–0.32) [10]
Volatile ammonia	0 [4]	0 [10]	0 [12]
Urea	0 [4]	0 [10]	0 [12]

Figures within parentheses give the range of values. These fluids are to be considered as having a natural variability and are not of constant composition. The above ranges exceed the experienced variability of the methods. Figures outside of parentheses are average values. Figures to the right of arrows are unusual values. Figures in brackets show the number of samples (sea water) or the number of animals analyzed. The various zeros are zeros for the method used. Analyses were done in duplicate or triplicate.

* Measured by Beckman and Cambridge meters. Sea water from the Bay was about 8.0; the running laboratory water was variable.

** Includes water of crystallization.

Some of these data were secured over several years, some in a single summer season, and some in shorter periods. For this reason they should be viewed only as general parameters. For critical quantitative work complete work-ups of individual animals should be made.

For this reason we have presented our data in Table I in the raw form of mM/liter. A crude generalized correction can be made by taking the blood protein as 50 g./liter.

1. *Phosphate.* Fiske-SubbaRow phosphate (method given by Hawk *et al.,* 1947) was absent from sixteen urines. Blood levels of eight lobsters were about 1.6 mg. % (range: 0.8–1.8). If exogenous inorganic phosphate (sodium salts mixed to a slightly alkaline pH) is injected, phosphate spills over into the urine. With blood levels of about 8 mg. %, phosphaturia occurred. The nephridium behaves toward inorganic phosphate as it does to glucose.

2. *Magnesium and calcium.* An ethylenediamine tetraacetic acid (EDTA) titration, developed by Dr. Martin Rubin (personal communication), was used. Calibration studies indicated that the method is serviceable if one discards all samples which do not titrate sharply. The method gives initially combined magnesium and calcium, then magnesium. Calcium is obtained by subtraction.

The analyses of Cole, Smith in Cole (1940) showed there is a marked partitioning between sea water and blood in *Homarus.* Robertson (1953) found a molal magnesium U/P ratio of 1.8, and a calcium U/P ratio of 0.64. Our data on fifteen lobsters give the following molar ratios (see above): blood/sea water: Mg + Ca, 0.37; Mg, 0.13; Ca, 1.68. Urine-plasma ratios were: Mg + Ca, 1.16 (range: 1–1.3); Mg, 1.7 (range: 1–2.6); Ca, 0.81 (range: 0.53–1). In most animals magnesium is concentrated in the urine and calcium is reduced. There are instances, however, where each ion is not affected (U/P ratio of one), and there was one animal where both the magnesium and calcium U/P ratio was one. In dilute sea water, the magnesium U/P ratio falls, and may drop to 0.8. In short, some qualification must be placed on the idea that the lobster's nephridium concentrates magnesium in the urine, and conserves calcium, although this is the usual situation.

The gills and carapace are relatively impermeable to magnesium. There was no elevation in blood or urinary magnesium in four lobsters placed for twelve hours in baths made of half sea water with magnesium chloride added to return the water to near its original equivalence. In lobsters naturally anuric, blood magnesium is at normal levels. There seems no tendency in these animals for magnesium to build up in the blood as might be expected if the gills were permeable inwardly to magnesium.

Since the lobster usually produces urine, it is losing magnesium with the urine. The daily urinary loss approximately equals the magnesium found in 5 ml. of sea water. The port of entrance seems to be the stomach. With urinating lobsters sea water is drunk intermittently with the food or on an empty stomach (see section on Intake and Output), and this sea water is absorbed. The stomach normally contains a concentration of magnesium greater than that of the blood, although in unfed lobsters empty stomachs are found frequently. Sea water placed in the stomach is slowly absorbed, and as shown below, the stomach fluid does not furnish the bulk of the fluid for the urine (24 ml./*diem*). It must not be thought that there is a steady rapid movement of magnesium-laden fluid into the blood from the stomach which would result in high levels of blood magnesium. The anuric animals mentioned above had empty stomachs.

With urinating lobsters, injected magnesium sulphate or chloride results in an increased magnesium excretion although the U/P ratios do not rise above those found normally; magnesium sulphate has a marked diuretic and then an anesthetic effect. Exogenous magnesium placed in the stomach is absorbed (see Intake and Output). In dilute sea water lobsters conserve magnesium. Four animals kept for 24 or 48 hours in 60–70% sea water had urinary levels lower than plasma levels. Blood levels remained within 1 mM/liter of the levels found with full sea water. Under these same conditions blood calcium fell more obviously, 2–4 mM, and the nephridium did not conserve calcium more than it did in full sea water.

It is obvious from Table I and from the above data that the nephridium has very modest powers in partitioning these two ions (Mg, Ca). Magnesium is lost more through the volume of urine flow than by nephridial concentration. Calcium is only weakly conserved. It should be noted that all the lobsters used were hard-shelled animals near the end of an inter-molt period.

Levels of blood magnesium can be changed without nephridial participation, at least on a short term basis. Blood levels were raised up to 33 mM/liter by placing magnesium chloride solutions in the stomach of anuric animals. Over a subsequent 11-hour period, blood magnesium fell to 10–21 mM/liter without urine production or without a rise in stomach magnesium. During this experiment, blood calcium levels remained unchanged. Either the magnesium was taken up by the tissues and carapace or it passed out through the gills.

3. *Sulphate.* Analyses were semi-quantitative. Known volumes of fluid were treated with known volumes of barium chloride in an ice bath, and the amount of precipitate was measured. The general picture for this ion is like that for magnesium. The gills and carapace are relatively impermeable to sulphate. After 18 hours, there was no elevation of blood or urinary sulphate in six lobsters placed in baths with elevated sulphate (Na, Mg). Blood and urine sulphate was elevated when sulphates were placed in the stomach. In 18 normal lobsters, the sulphate U/P ratio varied between one and two, but rose to four with small amounts of injected sulphate. With increased dosage up to blood levels of 300 mg. %, the U/P ratio fell toward one. Increased sulphate was markedly diuretic to urinating lobsters. Following the elevation of blood levels, the return to normal was slower than for magnesium; as with phenol red, days were required to effect normal blood levels. This seems to indicate that sulphate is lost primarily through the nephridia.

4. *Monovalent ions.* Natural values are given in Table I. Analyses for sodium and potassium were done by flame photometry. Chlorides were done by electrical conductivity measurements of silver nitrate titrations, or by a mercuric nitrate titration with s-diphenyl-carbazone as a visual indicator.

On the basis of a generalized calculated molality, using 50 g./liter protein, chloride ratios of sea water/blood and urine/blood are essentially one (1–1.01). This same ratio holds for serum dialyzed through Visking membranes against full strength and 60% sea water. Cole's data (1940) and Robertson (1949) give this same ratio. Under normal conditions of undiluted sea water the distribution of chloride in the blood and urine seems to be a passive one. This might be expected when one considers that the blood proteins have a negative charge and that there is no heavy concentration of a non-diffusible cation in the blood.

Cole (1941) has presented evidence that in dilute sea water the lobster can secrete chloride inwardly. Twelve lobsters whose blood/sea water molar ratio averaged 0.95 (sea water, 504 ± 9 mM/liter) were placed in dilute running sea water which did not exceed 394 mM/liter. Between 37 and 118 hours in this diluted sea water, and following an initial hemodilution, the blood/sea water ratio rose to 1.05–1.11 in five lobsters sampled.

In twenty-seven analyses of sea water, blood, and urine for normal lobsters, we found without exception that the molar blood/sea water ratio was below one, and the urine/blood ratio was above one. The millimolar differences between sea water and blood were about 25–35. Dialysis of serum of three lobsters (La, b, c) against sea water for 24 hours through Visking membranes gave the following chloride values in mM/liter: sea water, 511, 512, 518; La, 478, 478; Lb, 480, 485; Lc, 483, 487. Dialysis of serum of three lobsters (Ld, e, f) against dilute sea water gave the following values: dilute sea water, 308, 308, 311; Ld, 287, 287; Le, 288, 291; Lf, 287, 292. These values show that there is no capacity of the blood which tends to elevate blood chloride in situations where the blood is separated from full and dilute sea water by an inert membrane. Under dilute conditions, any marked rise in blood chloride can not be due to the blood itself or to an inert membrane effect.

Cole's experiment was repeated with seven lobsters (L1–7). For three lobsters (L1–3) pre-dilution values in mM/liter averaged: sea water, 510 ± 5; serum, 470 ± 5. Running dilute sea water varied between 310–338. After 72 hours, serum chloride values were: 370, 370; 357 ± 3; 355 ± 3. For L4–5, initial control values were: sea water, 500 ± 2; serum, 485 ± 5. Dilute sea water was 358–371. After 96 hours, serum values were 376, 380; 380, 384. For L6–7, control values were: sea water, 503 ± 7; serum, 485, 485; 477 ± 1. Dilute sea water was 378–392. After 116 hours in diluted sea water, serum values were: 420, 424; 412, 417. It should be noted that during the first twenty-four hours of dilution blood chloride falls below the above values, and subsequently rises.

In all the above animals in dilute sea water, the blood/sea water ratio was above one, an event which does not occur in full sea water or after dialysis with an inert membrane. It would seem that this elevation in blood chloride is due to some active process.

In all the above animals in dilute sea water, the urine chlorides were 20–33 mM/liter higher than the blood chlorides. When calculated on a molal basis, this difference disappears (see above). These data indicate that the nephridium is losing chloride at the elevated blood levels and the nephridium is not participating in the elevation of blood chloride nor is it helping to conserve chloride. In other Crustacea, nephridia are known to conserve chloride and the gills are known to secrete chloride inwardly. Here in the lobster, the nephridia clearly do not play such a role. While our data do not irrefutably establish the secretion of chloride, the most likely site for the agency effecting the elevation of blood chloride is the gills. The persistent elevated chlorides after 116 hours would seem to indicate that the higher blood chloride is not due to tissue chloride which has come out of cells but has not been carried away by the urine, diffusion, etc.

While it is clear that in full-strength sea water the distribution of chloride can be accounted for on a passive basis, the sodium picture is a bit more complex. On a molal basis there are about 60 equivalents more sodium in the plasma than in sea water. The sum of the equivalents of the major electrolyte cations of the blood (Na, K, Mg, Ca) accounted for about 99% of the cations of sea water. Since the data were worked up on an average basis the percentage figure should not be taken too literally, but merely to indicate there is not an unforeseen discrepancy between the cationic sum of blood and sea water (the blood and sea water are roughly isotonic; see below and Table I). The extra sodium of the blood can be accounted for on the basis of the exclusion of most of the magnesium of sea water from the blood. If one groups sea water magnesium and calcium, subtracts plasma magnesium and calcium and the equivalents bound to blood sulphate (arbitrarily taken as 10 mM/liter from data of Robertson and Webb, 1939 and Cole, 1940), one has about 50–60 equivalents which is the excess of sodium in the blood. Blood and sea water potassium are very close to each other (Table I).

The U/P partition is not so easily explained. There seems on a molal basis to be a persistent deficit (10–30 mEq.) of combined urinary sodium and potassium, largely sodium. Urinary potassium tends *irregularly* to be several milliequivalents lower than plasma (not higher); combined magnesium and calcium add only several milliequivalents to urine as compared to blood. Blood/urine potassium, magnesium, and calcium thus tend to cancel each other. Robertson (1949) gives a molal U/P) ratio for sodium of 0.99, indicating again a sodium deficit. It may be that since the urine is isotonic with the blood, there is an increased activity in the urine where the cations are free from the depressing influence of the blood proteins.

The above natural data and the following experimental data indicate that sodium chloride is freely mobile across the gill-blood and blood-urine barriers. On placing lobsters in dilute sea water (60, 70, 75, 80, 90%) the osmotic uptake of water, as judged by the gain in body weight, is completed in less than one hour, with a gain of 1–2% of body weight (18 animals). While this uptake results in hemodilution, the blood sodium chloride continues to fall, approaching the concentration of the external medium in twelve hours or less. The blood concentration after the first fifteen minutes of dilution falls in an almost linear fashion with a slope varying with the amount of external dilution. Within the first three hours the bulk of the sodium chloride to be lost is lost. Blood sodium chloride begins to asymptotically approach the external concentration but remains superior to the external concentration on a molar basis. On returning the lobsters to full-strength sea water, the concentration curves are not the reverse of the dilution curves. During the first hour the blood concentration rises very steeply and asymptotically approaches normal values in three to six hours. After injecting sodium chloride sufficient to raise blood levels 15–20 mM/liter, the blood returns to normal values in three hours. Under these altered sodium chloride loads, the urine reflects the blood concentration, rising or falling in sodium chloride concentration with blood concentration or dilution. In anuric lobster, blood sodium chloride adjusts to control values following the injection of hypo- or hypertonic saline within about three hours.

The above data suggest that while under conditions of external dilution, the lobster has the capacity to actively raise its blood chloride, under normal conditions of undiluted sea water, sodium chloride is passively distributed between sea water, blood, and urine. That is, normally there is no active process which is acting on sodium chloride as such. The amount of sodium in the blood can be explained as the amount in sea water plus the equivalents replaced for the sea water magnesium which is excluded from the blood. The discrepancy between blood and urine sodium is too small to support the idea that some active partitioning of sodium is being effected by the nephridium. Indeed, the dilution experiments indicate that the nephridium does not have a chloride-conserving mechanism.

Intake and output

While it is obvious that in the natural environment, the intake of organic chemicals is by mouth, it is not recognized that in the lobster sea water enters by mouth. The stomach capacity is about 10 ml. for a 500-gram animal. On eating, the food is diluted with a good deal of sea water. But even lobsters with empty stomachs will fill the stomach with sea water. This drinking is not due to fright or experimental handling. If the stomach is emptied by pipette, some animals will immediately fill the stomach with sea water. The stomach contents are never regurgitated unless the animal is in great distress. The stomach contents are always completely absorbed. The above observations come from dozens of lobsters made over five years and are not quick impressions. Many lobsters were followed at intervals for several days. It is not to be inferred that all non-feeding lobsters keep their stomachs full of sea water. The drinking is an intermittent affair, whose cause was not determined.

Since all substances mentioned in this report, with the exception of fish protein and lobster NPN which were not studied for this purpose, were found experimentally to be absorbed from the gut when placed in the stomach, the diet and imbibed sea water contribute to the internal electrolytic and non-elecrolytic content of the lobster. Since, too, the gills and carapace were found to be relatively impermeable to magnesium and sulphate, the stomach seems to be the principal port of entrance for these substances. Possible branchial uptake of calcium and phosphate was not studied, but these obviously enter with the diet. While water and monovalent ions do enter from the stomach, this route does not appear as the principal route (see below). The principal route seems to be the gills.

The nephridia and the gills seem to form the principal points of exit for the substances under discussion. The feces were not studied critically. Their fluid volume is small and only minute amounts of phenol red or bromsulfalein are lost with the feces. There seems little likelihood that the feces play any great role with the chemicals here discussed.

The concentrating powers of the nephridium have been discussed. The daily chemical loss through the nephridium depends not only on the nephridium's partitioning powers but also on the urine flow. For so-called normal lobsters the urine output is about 1 ml./hr./0.5 kg. (determined by hourly collections over 12-hour periods), with flows up to 4 ml./hr. with extreme diuresis, *e.g.* after injected magnesium sulphate. There are various degrees of oliguria and anuria; lobsters may be completely anuric for at least a month. Nephridial occlusion is not fatal to previously urinating lobsters over two- and three-week periods.

Contrary to possible supposition, the fluid volume of the lobster is not regulated by the volume containable within the exoskeleton. Fifteen to twenty-five ml. can always be injected into a normal animal (*c.* 0.5 kg.). The volume increase between emptiness and distension of the stomach (*c.* 10 ml.) and the nephridial bladders (*c.* 6 ml.) requires internal space into which these organs can expand. If one draws blood repeatedly, one sees directly a loss of blood volume which is not repaired in a few hours or even days to the initial volume. A lobster may be mobile and active, and yet from it blood can be drawn only slowly in contrast to the normal situation where blood can be drawn rapidly. Through the transparent ventral surface of the tail one can see the reduced blood volume.

In the normal animal there are about 24 ml., principally a sodium chloride solution, flowing through the animal and out as urine. This is at least several times the amount that is absorbed from the stomach. Indeed, urine at the above rate can be formed with lobsters with empty stomachs. It would appear that this sodium chloride solution must enter through the gills. Experiments with injected sodium chloride solutions (hypo- or hypertonic) with nephridial occlusion showed that water and sodium chloride can pass quickly (several hours) in either direction through the gills. Iodine also passes in either direction through the gills. Isotopic flux studies are necessary to define these parameters. The minimal net flux of water and sodium chloride is the urine flow, and this flow means that a sodium chloride solution is moving inwardly across the gills faster than it is moving outwardly across the gills.

The inward direction of this flow of a sodium chloride solution seems to be governed by the blood proteins. Anuria and oliguria were associated with low blood protein as determined by the copper sulphate specific gravity method or by the weight per volume of blood. A specific gravity below 1.029 accompanied poor urine formation. So-called normal specific gravities were in the 1.032–1.033 range. In some anuric lobsters, erratic transitory flows of urine could be induced by the infusion of hypo- or hypertonic solutions (Na, Mg, Cl, SO_4). The transfusion, however, of 10–15 ml. of serum from urinating lobsters into anuric lobsters induced permanent urine flows, *i.e.,* the regular production of urine over a subsequent one week's test period (8 animals). Osmolar measurements by the Thermistor method (Table I) always gave isotonic or slightly hypertonic, never hypotonic, values for the blood as compared with sea water. The withdrawal of large amounts of blood from urinating lobsters, *e.g.* 0.1 of the blood volume (see Burger and Smythe, 1953), did not check urine formation, apparently because the blood did not become markedly diluted. With a 1200-gram lobster with a blood specific gravity of 1.032, the removal of 24 ml. of blood did not check urine formation nor was the specific gravity of the blood lowered 1.5 hours later. With a 650-gram animal, blood protein sp. gr. 1.0335, the specific gravity one and five days after the removal of 10 ml. of blood was 1.032.

Urine formation could not be correlated with hemocoelar blood pressure, a rise in which is accompanied by a rise in arterial pressure (Burger and Smythe, 1953). Hemoconcentration, resulting from keeping lobsters in the air, suppresses urine formation. In view of the above transfusion experiments, it would appear that the non-diffusible molecules of the blood draw in water principally through the gills, and this water is bailed out by the nephridium as urine. With this water moves sodium chloride. Ions to which the gills are not readily permeable, as well as some sodium chloride solution, enter from the stomach and leave through the nephridium. The nephridium keeps the blood volume below the fluid capacity of the shell. The constant nephridial removal of water from the blood should slightly raise the osmotic pressure of the blood. Since the blood circulates rapidly through the gills (Burger and Smythe, 1953), a very slight gradient should be enough to extract water from the external medium. The nephridium, however, is not merely an organ for maintaining fluid volume. It is capable of secreting some organic and inorganic substances and of restraining completely or partially other constituents of the blood. In short, it acts like a kidney. The all-over pattern seems to be one where substances are carried away by a high urine flow rather than by a powerful concentration of secreted substances. Only with phenol red, and only with very low blood concentrations was nephridial concentration marked. Since anuric lobsters live for at least a month without eating or drinking and since electrolytes such as magnesium do not build up in the blood, there is presumably no net inward flow of fluid as is found in the urinating animal. If the nitrogenous waste is ammonia, this can be lost through the gills. While there is no evidence that the lobster forms urea, exogenous urea is lost through the gills, and not through the nephridium. The absence of any volatile ammonia in the urine and the lack of any clear U/P difference in NPN make it dubious that the kidney is concerned with primary nitrogen excretion, although the occurrence of a persistent blood NPN indicates nitrogenous compounds which are not readily diffusible through the gills and which are removed in the urine. The gills, stomach, and nephridia form an interlocking system of entrance and exit, each with individual capacities.

This report gives no new information on the mechanism of urine formation. That the blood and urine are absolutely isosmotic (Table I) would be expected when one sees the extreme delicacy of the walls of the large nephridial bladders. Despite this delicacy, substances such as phenol red can be held at higher than blood concentrations. Pressure from the nephridial fluid must distend these bladders and one can imagine a small isosmotic filtration from bladder to blood, or the reverse when the blood pressure is raised.

From the work of Peters (1935) it is assumed that urine is formed by filtration. The ready passage of fish blood proteins and of inulin supports the filtration idea. Histologically, the nephridium seems more comparable to the aglomerular fish kidney where filtration does not occur. The nephridial arteries lie behind a wall of glandular epithelium and there seems to be no glomerulus-like structure or arrangement. This problem is worthy of further study.

SUMMARY

1. A method is given for safely securing repeated evacuations of the nephridial bladders of the lobster, *Homarus,* thus permitting experimental analysis of nephridial function.

2. Routine chemical analyses for blood, urine, and sea water are given.

3. Experimental analysis shows that the nephridium can concentrate in the urine phenol red, para-aminohippurate, magnesium, and sulphate. At normal blood levels, it completely excludes glucose and Fiske-SubbaRow phosphate. Phlorizin blocks glucose retention. With high blood levels, the secretory or exclusion powers of the nephridium are swamped. Calcium is partially excluded from the urine. The nephridium is indifferent to inulin, bromsulfalein, dogfish hemoglobin and plasma protein, sodium and chloride. The ability of the digestive gland to concentrate phenol red and bromsulfalein in the digestive juice is noted.

4. Exogenous urea is lost through the gills.

5. The gills and carapace are relatively impermeable to magnesium and sulphate, and to phenol red, but are freely permeable to water, sodium chloride, and to exogenous urea.

6. In full-strength sea water, the distribution of sodium chloride between sea water, blood, and urine seems to be passive. In dilute sea water, experiments indicate that blood chloride is elevated in some active fashion, presumably by the gills. The nephridium, however, does not aid in the conservation of chloride. Chloride is lost at the elevated blood level. The nephridium does not seem to have powers to deal actively with sodium chloride.

7. The normal urine flow is approximately 1 ml./hr./0.5 kg., with wide variations. Water and sodium chloride for this flow enter largely through the gills although there may be intermittent contributions from the stomach. Magnesium and sulphate enter largely through the stomach. Calcium, in addition to that of sea water, is in the food. The lobster intermittently drinks sea water with food or on an empty stomach. The stomach contents are absorbed completely but not rapidly enough to furnish the fluid for the bulk of the urine flow. The daily

urinary excretion of magnesium roughly equals the magnesium found in 5 ml. of sea water. All test substances entered the blood from the stomach. In any study of sea water and blood, the gastric contribution to the chemistry of the blood, even for electrolytes, must be considered.

8. Lobsters from commercial pounds are frequently oliguric or completely anuric. Inability to form urine is not lethal, at least over a one-month period. Erratic transitory urine flows can be induced in anuric lobsters by the injection of various saline solutions. Normal urine flow can be induced in anuric lobsters by the transfusion of serum from one lobster to another. Apparently, substances such as blood proteins which are not diffusible through the gills draw in water which is bailed out by the nephridia. Osmolar measurements found the blood isotonic or slightly hypertonic, never ~~iso~~HYPOtonic, to sea water.

9. While the nephridium has a range of capacities for dealing with individual substances (secretion, exclusion, partial exclusion, and a lack of partition power), its secretory capacities are not great and are masked easily by elevated blood levels. Nephridial removal of substances from the blood depends more on a flush-out principle, using large urine flows, than upon secretory powers. Some substances such as exogenous urea are lost by the gills and not by the nephridium. Together, the nephridia and the gills form an excretory system, each with individual capacities.

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BODY GROWTH VERSUS SHELL GROWTH IN BALANUS IMPROVISUS[1]

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The acorn barnacles are unique among the arthropods in that the body is permanently enclosed by, and separated from, the outer shell of calcareous plates. The chitinous exoskeleton of the body, the inner chitinous lining of the mantle adjacent to the shell-secreting tissues, and the opercular hinge are shed at regular intervals of two to three days in *Balanus improvisus* (Costlow and Bookhout, 1953) and in *Balanus amphitrite niveus* (Costlow and Bookhout, 1956). The calcareous shell, which grows continuously, is not shed and apparently is not affected by the molting cycle of the body.

Numerous papers have appeared which deal with the secretions of the endocrine organs in relation to molting of Malacostraca. These have been reviewed recently by Knowles and Carlisle (1956). Other research workers have been concerned with the subdivision of the molting cycle (Carlisle and Dohrn, 1953; Drach, 1936, 1939; Hiatt, 1948; and Kincaid and Scheer, 1952). Most of these studies have been confined to two groups, the Brachyura and Astacura, and have not been primarily concerned with actual growth. Relatively few investigators have considered the increase in size following ecdysis. Tait (1917) studied molting in the isopod, *Ligia oceanica,* and makes reference to size after molting. Olmsted and Baumberger (1923) worked on the form and growth of grapsoid crabs. Marshall (1945) noted that molting can occur without growth in *Panuluris argus* maintained in live cars and Dawson and Idyll (1951) confirmed these observations from studies on tagged individuals. In all of these studies direct measurements of the exoskeleton could be made, a procedure which could not be followed in the barnacles without removing the body and thereby killing the animal. It is not surprising, therefore, that most studies on growth in barnacles have been confined to the shell.

The rate of shell growth has been studied for several species of barnacles, under both natural and experimental conditions. Barnes (1955) described the growth rate of *Chthamalus stellatus* and Barnes and Powell (1953) studied the effect of varying conditions of submersion on shell growth in *Balanus balanoides* and *Balanus crenatus.* Crisp (1954) described morphological changes in the shell which are associated with differences in yearly growth rates of *Balanus porcatus.* The relationship between the daily increments of the continuously growing shell and the growth rate of the body, following a series of consecutive molts, is not known for any of the acorn barnacles. In order to obtain data over a series of consecutive molts of the same individuals it is theoretically possible to measure the exuvia, or

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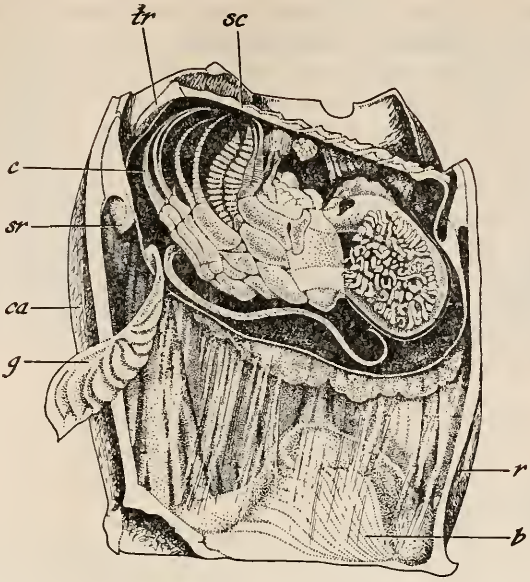

Figure 1. Sagittal view of a typical barnacle, with left side of shell removed, showing relationship between internal chitinous covered body and external calcareous shell.

Abbreviations

c —cirri of body
ca—carina: posterior plate of shell
b —basis
g —gill
r —rostrum: anterior plate of shell
sc—scutum: anterior opercular plate
sr—shell ring: calcareous ring to which opercular plates are attached by opercular hinge
tr—tergum: posterior opercular plate

some portion of it, following ecdysis. Since the shed exoskeleton tends to wrinkle, accurate measurements of total length or width are not possible. The mandibles and maxillae, however, are sufficiently rigid to retain their shape and size and, as will be demonstrated, reflect the per cent increase of the entire body at molting.

The objectives of this study were three-fold: one, to determine whether or not body growth always accompanies an ecdysis; second, to follow the increase in body size, as represented by two mouth parts, through consecutive molting periods; and, third, to compare the relative increase in size of the body with that of the shell.

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Methods

Balanus improvisus which had metamorphosed from the cyprid during the previous 24 hours were obtained from pyralin plates suspended on racks beneath the laboratory dock. Twenty barnacles were maintained individually in plastic compartment boxes containing *Chlamydomonas* sp. at 25° C. under daylight fluorescent lights. Each compartment was examined twice daily for the presence of molts. When exuviae were found they were removed and mounted on glass slides. In young barnacles the mouth parts, maxillae and mandibles, were dissociated from the remainder of the exoskeleton by repeated manipulation of the cover-slip. In older barnacles it was necessary to dissect out these parts with insect pins or glass needles. The width of the mouth parts, at the base of the spines or teeth, was then measured with an ocular micrometer mounted in a compound microscope. If the actual molting process was observed, the exuvia was removed as soon as possible, the mouth parts measured, and then returned to a depression slide containing the culture medium. Twenty-four hours later the maxillae and mandibles were measured again to determine if any changes in size had occurred.

Daily measurements were also made of the barnacle shell, rostro-carinal length and lateral-lateral width, with an ocular micrometer mounted in a dissecting microscope. The barnacles were observed and measured over a period of 60 days, an average of 20 molting periods.

A second series of 20 barnacles, of varying size and age, was obtained from the harbor. The entire body was removed from the shell and the widths of the thorax, maxillae, and mandibles were measured. The thorax was measured to determine if, in living barnacles, a relationship exists between the size of the mouth parts and a major body dimension, or if growth of the mouth parts is differential.

RESULTS AND DISCUSSION

The body of a sessile barnacle is so completely enclosed by a calcareous shell (Fig. 1) that direct measurements of the body cannot be made over a series of molts. To obtain data on body growth in relation to molting it was necessary, therefore, to resort to a measurement of mandibles and maxillae of a consecutive series of exuviae. It must be established, however, whether the increase in size of an appendage, or a distinct portion of the animal, reflects the increase of the body or represents a differential growth rate which is restricted to a specific part. The results obtained from measurements on whole bodies and mouth parts of 20 living barnacles taken at random from the harbor indicate that there is a definite correlation between the width of the thorax and the width of the maxillae. A ratio of 6.271 ± 0.104 was found in barnacles of varying size and age. A simple correlation coefficient of 0.922, significant at the 1 per cent level, was determined from the sample. This value exceeds that given by the standard tables at 18 degrees of freedom and indicates that 85 per cent of the variability in thorax size is associated with concomitant variability in maxillae size, leaving 15 per cent attributed to other factors including chance. Thus the maxillary size, as well as per cent increase at molting, is not differential but reflects the increase in size of the body.

Even though the barnacles were examined every 12 hours, it was not possible to observe the actual time of molting for each barnacle. Thus there was the possibility that changes in size of the exuviae could occur from the time of ecdysis until actual measurement. To determine if shrinking or swelling of the shed mouth parts occurred, they were measured immediately after molting, when the process was

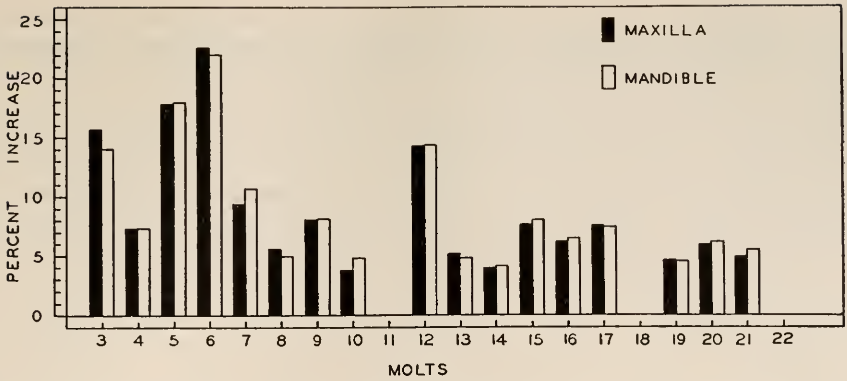

FIGURE 2. Per cent increase of maxillae and mandibles of one *Balanus improvisus* from the 2nd to the 22nd molt.

observed, and again after 24 hours' immersion in the culture medium. No change in size was found in the mouth parts after 24 hours and, inasmuch as all measurements were made within 8 to 12 hours after each ecdysis, the recorded size accurately indicates the width of the mouth parts prior to molting.

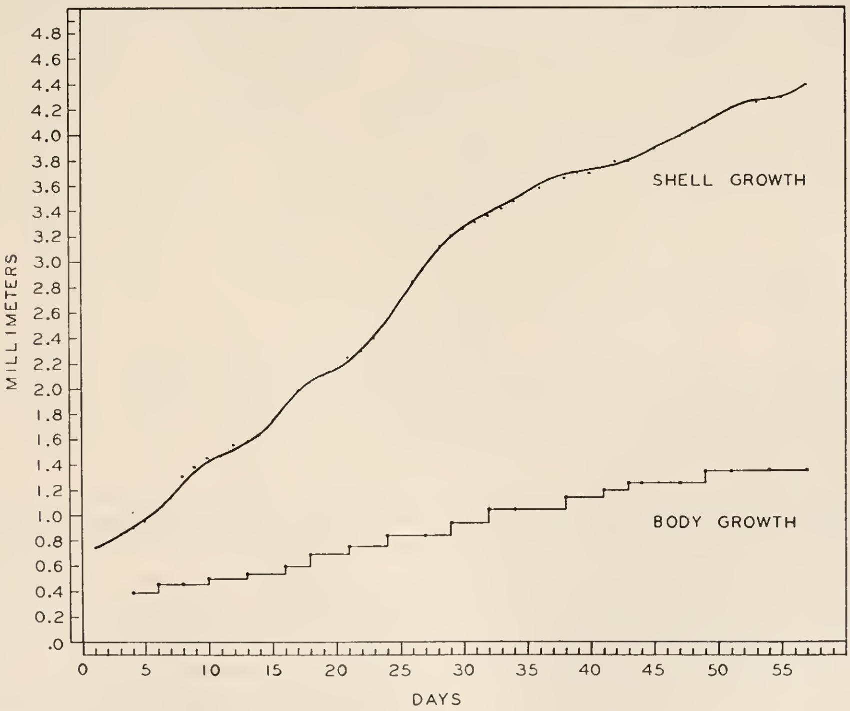

FIGURE 3. Comparison of shell growth and body growth over 23 molting periods for one *Balanus improvisus*. Figures for maxillae size, representing increase in body size; × 10^{-1}.

The mandibles, while more difficult to measure accurately, are approximately twice the width of the maxillae. This ratio is also maintained in barnacles of varying size and further supports the hypothesis that the size increase of the maxillae is not differential but reflects the size increase of the entire body.

Body growth. Figure 2 compares the per cent increase in size of the maxillae and mandibles from the second to the twenty-second molt. It will be noted that the per cent increase corresponds closely at each molt. That is, if the mandible increases greatly following one molt, the maxillae increase approximately the same per cent and similarly, if one shows no increase at molting, no increase is found in the other. Figures 2 and 3 indicate that increase in size of the maxillae does not necessarily accompany molting. There are several periods, ranging from one to three consecutive molts, when no measurable increase in maxillae size occurred (Fig. 2, molts 11, 13, 18, and 22; Fig. 3, molts 21, 22, and 23). Tait (1917) reported that no discernible increases in size occurred when *Ligia oceanica* molted, but he did not actually measure the pre- and postmolt animals. Carlisle (1956) found this to be true only when the animals were starved. Marshall (1945) reported the absence of growth after molting in *Panuliris argus* maintained in live cars and Travis (1954) also found that increase in length did not necessarily accompany molting in *P. argus* maintained in the laboratory. That this condition cannot be attributed solely to sub-optimal environment of the live cars and the laboratory is evidenced by the work of Dawson and Idyll (1951). Their study of tagged individuals, recovered from the area around the Florida Keys, showed an absence of growth in some individuals and 7 cases of "negative growth." The latter, however, were attributed to faulty measurements. Lloyd and Yonge (1947), working with female *Crangon vulgaris* under laboratory conditions, found that normally there is an increase in size after molting. No increase occurred, however, at the molt leading into the egg-laying condition. Figure 3 gives the increase in maxillae size for one of the 20 *B. improvisus* taken at random. Molting without growth, however, was observed in each of the 20 barnacles studied, although not necessarily at the same time or molt. This was true even though the same quantity of food was supplied each day.

As shown in Figure 3, the increments in maxillae size over a period of 20 molts are variable. In some instances, a slight increment was followed by a molt which produced considerable increase in size. Olmsted and Baumberger (1923) found that *Pachygrapsus crassipes* does not increase by the same fraction at each molt and that the increment gradually diminishes as the crabs become larger. Lloyd and Yonge (1947) also noted a decrease in size increment at molting with increase in over-all size but attributed it to egg production. These observations were apparently made on molts of various sized individuals and not consecutive ecdyses for the same animals. It does apply, however, to size increase in the maxillae of *B. improvisus.* Olmsted and Baumberger (1923) attribute some reduction in increment at molting to the decrease in molting frequency with age in *P. crassipes.* Costlow and Bookhout (1953) found that the frequency of molting in *B. improvisus* continued to be on the average of two to three days through the first 40 molts. Thus, reduction of molting frequency with age is not a factor which influences the gradual reduction in size increment observed for *B. improvisus* during the first 20 molting periods.

Shell growth. In contrast to the body, which may grow only at the time of molting, the calcareous plates of the shell grow continuously. Figure 3 compares the growth rates of shell and maxillae for one barnacle. As indicated previously, there were periods up to three molts when no increase in maxillae size occurred. During this same interval of time the shell continued to grow. Costlow and Bookhout (1953), comparing shell growth of *B. improvisus* in the laboratory with that found in the natural environment of the harbor, found that while the two rates followed the same general curve, growth in the laboratory was approximately one-third that found in nature. Thus, while not optimal, laboratory conditions will support growth of the shell and the absence of body growth cannot be attributed to starvation.

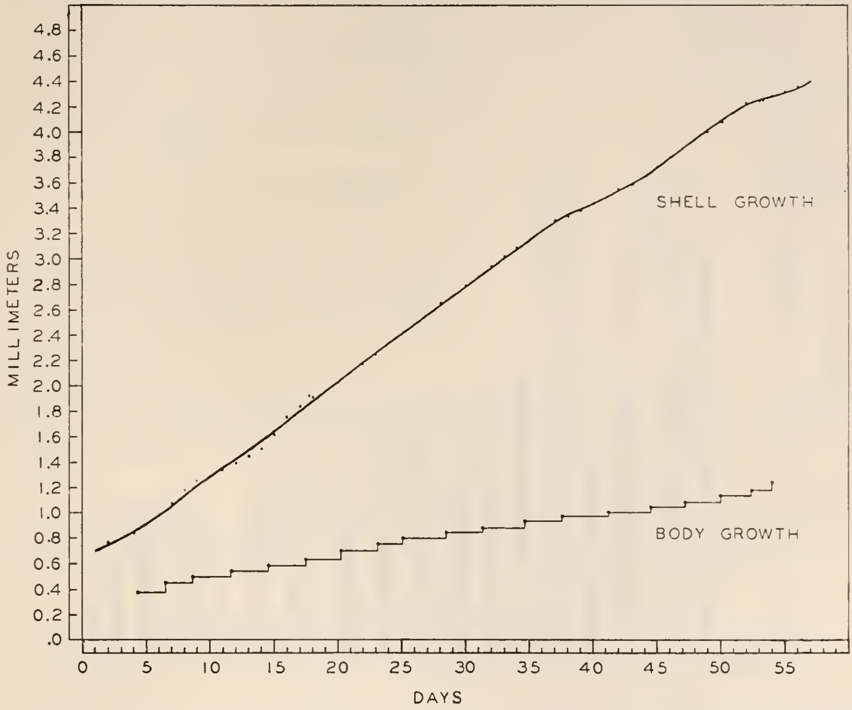

FIGURE 4. Comparison of average shell growth and average body growth, as indicated by measurements of the maxillae, for 20 *Balanus improvisus* over a period of 20 molts. Maxillae size × 10^{-1}.

Figure 4 shows the average increase in size of maxillae and shell for 20 barnacles. It will be noted that the initial ratio between maxillae and shell sizes is not maintained over any great length of time. As the body can grow only by molting, it might be expected to show an increase in size which would correspond with shell growth for that particular intermolt period. As shown in Figure 5, however, the per cent growth of the maxillae rarely equals the per cent accumulated shell growth during that intermolt period. With the exception of the third molt, the total per cent shell growth during the two- to three-day intermolt period was always higher than that exhibited by the maxillae. If this trend were to continue it would result in a large shell enclosing a relatively small body. The changing ratio shown in Figures 3 and 4 is compensated in part by changes in opercular plate level at the time of molting. In a newly set barnacle the opercular plates occupy an extreme apical position. At each molt a new opercular hinge is secreted and the opercular plates gradually become more basal. While this tends to reduce the internal volume of the shell, a large shell and relatively small body are not totally incompatible when certain other factors are considered.

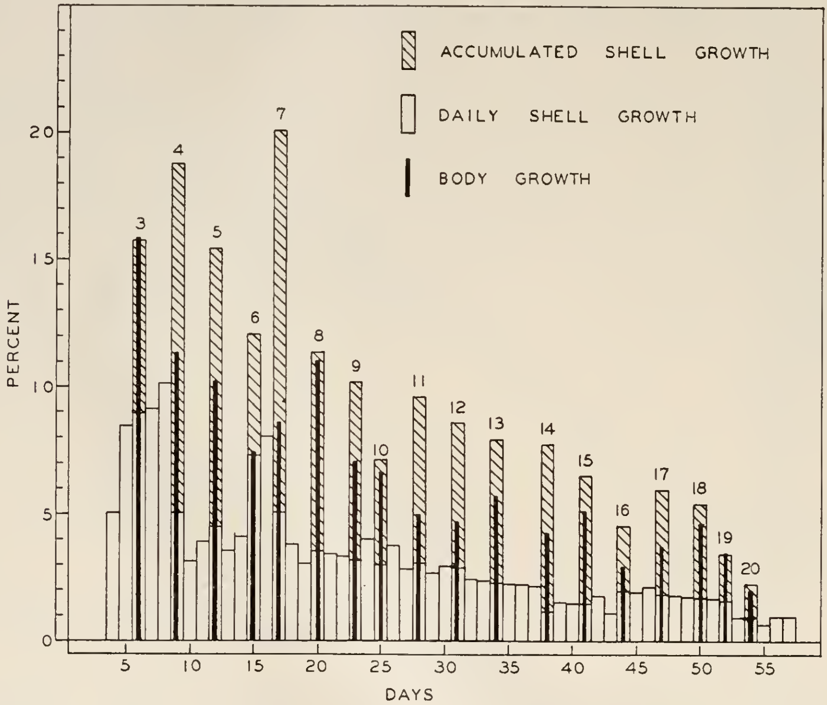

FIGURE 5. Comparison of average daily per cent shell growth, average accumulated per cent shell growth during each intermolt period, and average per cent body growth for 20 *Balanus improvisus* over 20 consecutive molting periods.

B. improvisus retains the fertilized eggs within the mantle cavity until the time of hatching, when the nauplii are expelled through the opercular orifice. While actual figures are not available, Bousfield (1955) has estimated that *B. improvisus* retains from 1,000 to 10,000 eggs during each breeding period. These embryos, forming lamellae on both sides of the body, require space in the mantle cavity between the body and the shell-secreting mantle. In a young barnacle the mantle, covering the internal surface of the developing shell, occupies considerable space and, with the body, leaves little room for the mantle cavity. Because of the intricate construction of the shell (Costlow, 1956) it has not been possible to accurately measure the volume which is actually utilized by the body at different ages. If the original ratio between the body and shell sizes were maintained, however, the size of the mantle cavity might not be sufficient to allow retention of the large numbers of embryos.

Summary and Conclusions

From daily measurements of shell and portions of the shed exoskeletons of 20 *Balanus improvisus* over 20 molting periods, and comparison of mouth part size and body size of barnacles taken from the harbor, the following conclusions may be made:

1. A ratio of 6.271 ± 0.104 exists between the width of the thorax and the width of the maxillae in barnacles of varying size and age taken from the harbor. Thus, the maxillae, which retain their form and size in the shed exoskeleton, are not subject to differential growth and may be used as an index of body size increase. The mandibles, approximately twice the width of the maxillae, may also be used as an index of body size.
2. Growth of the body, as indicated by measurements of maxillae and mandibles, does not necessarily accompany ecdysis. The absence of growth in mandibles and maxillae occurs frequently and may extend over periods of three molts.
3. The increase in size of the mouth parts is variable and tends to become smaller as the over-all size of the barnacle increases.
4. Growth of the calcareous shell occurs even when there is no increase in size of the mouth parts.
5. The accumulated shell growth during the two- to three-day intermolt period is usually greater than the size increment of the maxillae and mandibles at molting. The original ratio between body size and shell size is not maintained as growth occurs.
6. The hypothesis is presented that the resulting large shell and relatively smaller body of an adult barnacle provide a mantle cavity sufficiently large to accommodate the developing embryos.

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FACTORS AFFECTING TERMINAL GROWTH IN THE HYDROID CAMPANULARIA[1]

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The experiments reported here pertain to the limitation of terminal growth in the hydroid *Campanularia flexuosa.* The general problem of growth limitation, and specifically of terminal growth, has been examined for many different organisms. A review is impossible in a brief paper. Few general unifying concepts seem to have been established clearly: we are still in the stage of assembling the necessary facts.

Three considerations led to these particular experiments. First, the fact that each species of hydroid has a stem of characteristic and fairly definite height suggests the presence of limiting factors which must either vary from species to species or have a variable effectiveness. Secondly, an earlier experiment (Crowell, 1957) had shown that terminal growth is very sensitive to nutritional level, and also that the effects of lowered nutrition are more striking the older the stem. This assured us that the terminal growth zone is not autonomous but has a dependence upon events and conditions beyond itself. Thirdly, there was a paradox in our observations: well-fed young colonies added one hydranth per day. The internodes or distances between adjacent hydranths are (almost exactly) one mm. long. A stem 35 days old should be expected to have 35 hydranths and be 35 mm. tall. Yet our older colonies in the laboratory had only 20 hydranths at the most, and were only about 20 mm. high. Likewise, in nature, stems of this species are rarely as tall as 30 mm. and usually are much shorter.

This discrepancy and the general considerations led to the following questions. Is a slowing down inevitable? Is it gradual or abrupt? Does the ability to grow finally cease entirely? Can experimental procedures be used which eliminate the inhibiting factors? Clear answers should give clues to the nature of such factors.

Figure 1 and the following remarks will enable a reader who is unfamiliar with hydroids to visualize the pattern of growth and understand the terminology employed. A colony has an attached branching stolon system from which stems arise. The first hydranth of a stem, 1 in Figure 1, is produced by upward growth from the stolon. An apical growing zone produces an internode of the main stem, then a pedicel, and finally a hydranth bud. After this there is renewed proliferation from the first internode to produce the next internode, the pedicel of hydranth 2, and hydranth 2. This sequence is continued as hydranth after hydranth is added terminally. The increasing height is due not to continuous apical growth but rather to a renewal of proliferative activity by a succession of zones, each of which, at the time of its activity, is in a location just proximal to the most distal hydranth, (GZ in Fig. 1).

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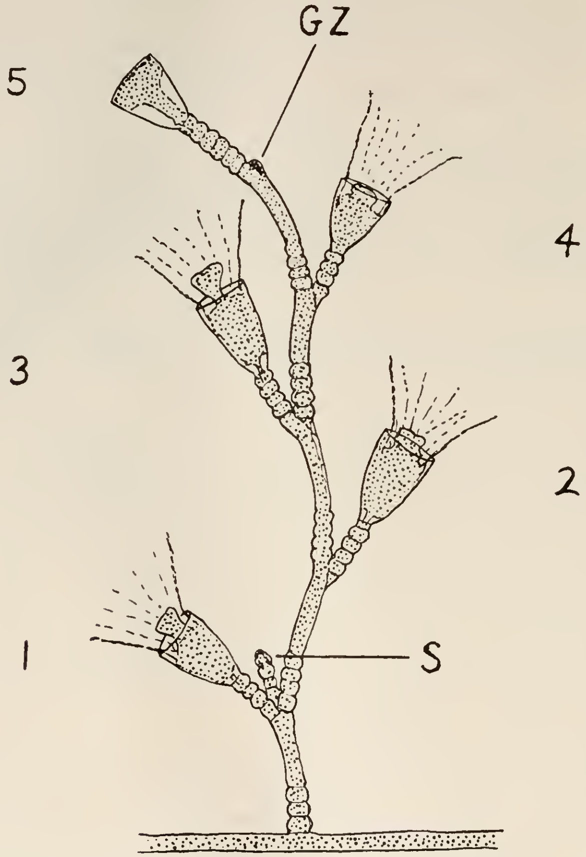

FIGURE 1. A young upright showing the pattern of growth and the location of the terminal growth zone. GZ, terminal growth zone; S, secondary growth which may produce a branch or a gonangium; 1—5 the designations of the nodes and of the primary hydranths or positions.

In *Campanularia* both gonangia and lateral branches develop later from some of the crotches (S in Fig. 1). Also, hydranths live for only about a week, after which they undergo regression and are replaced by a new hydranth at the same location (Crowell, 1953). These secondary developments may be relevant to the problem of apical growth since they must be in competition with the apical growth zone for available nutrition, and perhaps also in less obvious ways. In this report, however, they have been disregarded.

The term *stem* (or *hydrocaulus*), strictly speaking, means only the stem itself. There is no technical term for the stem plus all the hydranths, side branches, and gonangia which it bears. We call these *uprights:* that which grows up from, or away from, the stolon. An approximate botanical equivalent is *shoot.* The term *position* is used to indicate the primary hydranths or nodes. Each is numbered as in Figure 1. Distances along an upright are described in number of positions.

The colonies used for experimentation were developed from cuttings from cultures originally obtained at Woods Hole two years earlier. These grew on microscope slides, in running filtered sea water cooled to 19° C. Colonies were fed twice a day by placing them in a dense suspension of newly hatched brine shrimp (*Artemia*) for 5–10 minutes, a procedure which provides nutrition for maximal growth.

The Rate of Terminal Growth

Since terminal growth is not continuous, but is a consequence of renewal of activity by tissues just proximal to the last hydranth, the best measure of rate which we have been able to devise is the time in hours from the *cone stage* of the terminal hydranth to the same stage of the next terminal hydranth. The *cone stage* may be thought of as a hydranth bud; it represents a point in the development of a hydranth at which cellular proliferation is about finished and differentiation is commencing. During one three-day period, observations were made every three hours to determine exactly the time difference between each of the developmental stages of a hydranth. With this information it was possible, in the principal experiments, to make observations once each day and then convert these to the time at which the cone had been or would be present. Since this method depends upon the assumption that once development of a hydranth is initiated it develops at a constant rate, the evidence for this needs to be set forth.

In the series of close observations made to establish the normal times for development, we found no significant differences between old and young uprights except in the period of delay prior to the beginning of development. The same is true when specimens at different nutritive levels are compared (Crowell, 1957). Regrettably, the earlier report of Lund (1923) was overlooked in that paper. Lund gives detailed measurements and states (p. 86) that "The sequence in the formation of polyps on isolated internodes of *Obelia* is not due to a difference in rate of regeneration, but is due to a difference in the period of delay between cutting and initiation of the regeneration process. The rate of regeneration (growth) of polyps is the same in basal and apical internodes." *Obelia* is almost identical with *Campanularia* in its growth. An analogous condition is reported by Steinberg (1955) in *Tubularia,* a hydroid quite different from *Campanularia.* He finds that the difference in time of hydranth regeneration under varied conditions is in the preparatory phase, but not in later stages.

In the first experiment several colonies with a large number of uprights were used. Each day a record was made of the stage of development of the terminal hydranth of each upright. Every 2–3 days new stolons were removed so that each colony consisted of only about 20 of the oldest uprights. At the end of 5 weeks this trimming was stopped to permit the growth of a "crop" of young uprights. When the latter had reached a height of about 10 positions, both they and the older uprights were used for the second series of experiments described later on.

On two occasions during the experiment, when the *Artemia* supply was known to be sub-maximal, old uprights which had been adding a new position every

30–35 hours would suddenly require from 72–144 hours. The older the upright, the more pronounced was this slowing down. With equal suddenness, however, as soon as they were returned to maximum feeding, the time shortened to 30–35 hours. Large values obtained during times of food depletion were not included in the averages.

The rate of terminal growth, measured in hours, from cone stage to cone stage, is shown for each position on the uprights in Table I and in Figure 2. Rate, strictly speaking, should be expressed as quantity per unit time, or reciprocal of hours. The conversion seems unnecessary here. A few uprights were kept be-

TABLES I–III

Hours required for the development of a new terminal hydranth at each position on an upright. In each table is shown the position, the number of cases, the mean, and the standard deviation. The mean values are plotted in Figure 2

Table I. The main series of observations; —● in Figure 2.

Position	n	M	SD
1	48	24.9	4.1
2	50	23.8	4.9
3	50	22.1	3.7
4	53	23.6	4.8
5	54	23.8	5.4
6	53	24.5	4.7
7	51	24.4	4.2
8	52	24.9	4.7
9	47	26.2	4.2
10	42	27.3	3.7
11	36	26.1	3.6
12	37	28.6	4.0
13	42	29.2	4.2
14	43	30.0	5.7
15	45	30.8	4.5
16	39	30.8	4.9
17	31	31.3	4.3
18	33	32.4	5.5
19	38	33.4	6.0
20	35	35.6	5.1
21	39	35.6	5.8
22	36	32.0	4.9
23	41	33.8	6.6
24	47	32.4	4.5
25	49	32.6	6.0
26	48	33.9	7.7
27	45	32.8	10.7
28	36	32.9	5.8
29	28	31.6	6.1

Table II. The old controls (0:con) during the experimental period; —○ in Figure 2.

Position	n	M	SD
31	10	28.6	3.5
32	10	32.0	4.4
33	10	30.0	3.1
34	9	32.6	4.9
35	8	30.5	4.0
36	7	32.9	2.7
37	6	31.8	4.4
38	4	32.0	2.5

Table III. Young controls (N:con) before and during the experimental period; —× in Figure 2.

Position	n	M	SD
1	12	27.3	5.3
2	17	23.4	4.9
3	18	21.6	3.4
4	18	21.7	4.1
5	18	22.5	3.1
6	18	22.0	2.0
7	18	22.4	3.4
8	18	22.4	2.4
9	18	23.3	3.0
10	17	22.9	2.0
11	18	25.4	3.7
12	18	25.1	2.8
13	17	27.1	3.9
14	18	29.1	4.5
15	17	29.0	5.4
16	16	28.6	3.3
17	16	30.1	3.8
18	12	30.1	4.4

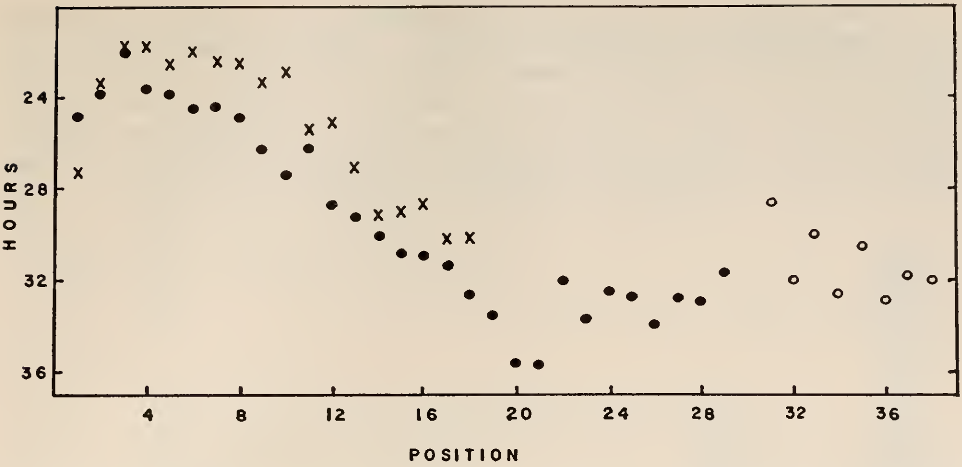

FIGURE 2. The rate of development of terminal hydranths, expressed as number of hours from one cone stage to the next, for each position on a stem. ●, main series of observations. ○, a few of the same stems continued (0: con). X—a second series of observations (N: con). Data in Tables I–III.

yond the 30th position and used as controls in the subsequent experiments. These are plotted as 0 in Figure 2 and the data are given in Table II. Also, a second series of values was available for young uprights and these are shown by X in Figure 2 and are presented as Table III. The slightly higher values obtained in these later observations are probably a reflection of some slight, but unrecognized, improvement in conditions. In Figure 2 three periods are identifiable: During the first 8–10 days one new hydranth is added each 24 hours; then the rate becomes slower until about 20 positions are established; after this there is a period of steady rate at about 33 hours. How long this might continue no one knows. The oldest stems, at about 60 days, were taller than any we have seen either in nature or in our ordinary cultures.

Amputated Uprights and Sections of Uprights

The following experiments were designed to show the terminal growth rate when the relationship of the terminal growth zone to the rest of the upright and to the colony is altered. The uprights whose history had been closely followed for 5 weeks, and the newer uprights which were 8–13 positions high, were cut according to the scheme shown in Table IV and Figure 3. Those sections which were detached were held in place at the edge of slides by a thread. Their proximal ends, as well as the basal ends of any lateral branches, were ligatured with a single strand of Dacron to prevent growth at all but the distal end.

Measurements were made for the time from cone stage to cone stage for the four hydranths produced terminally on each upright of the control groups. In the experimental groups the hydranth develops from the cut end of the upright and the time is measured from the moment of cutting to the cone stage. The times for the three successive hydranths, which develop from the zone of prospective growth, are measured as before, from cone stage to cone stage.

In these experiments the distal cut end sometimes failed to produce a hydranth, but instead produced an indeterminate growth similar to a stolon. These are called *free* (*i.e.,* not attached) *stolons.* When they developed we cut them off. Following such amputation, a free stolon sometimes developed again and sometimes the new growth was a hydranth. This complicates the analysis and makes it necessary to report both the rate of hydranth development when it did occur and also the incidence of hydranth development as compared with that of free stolons.

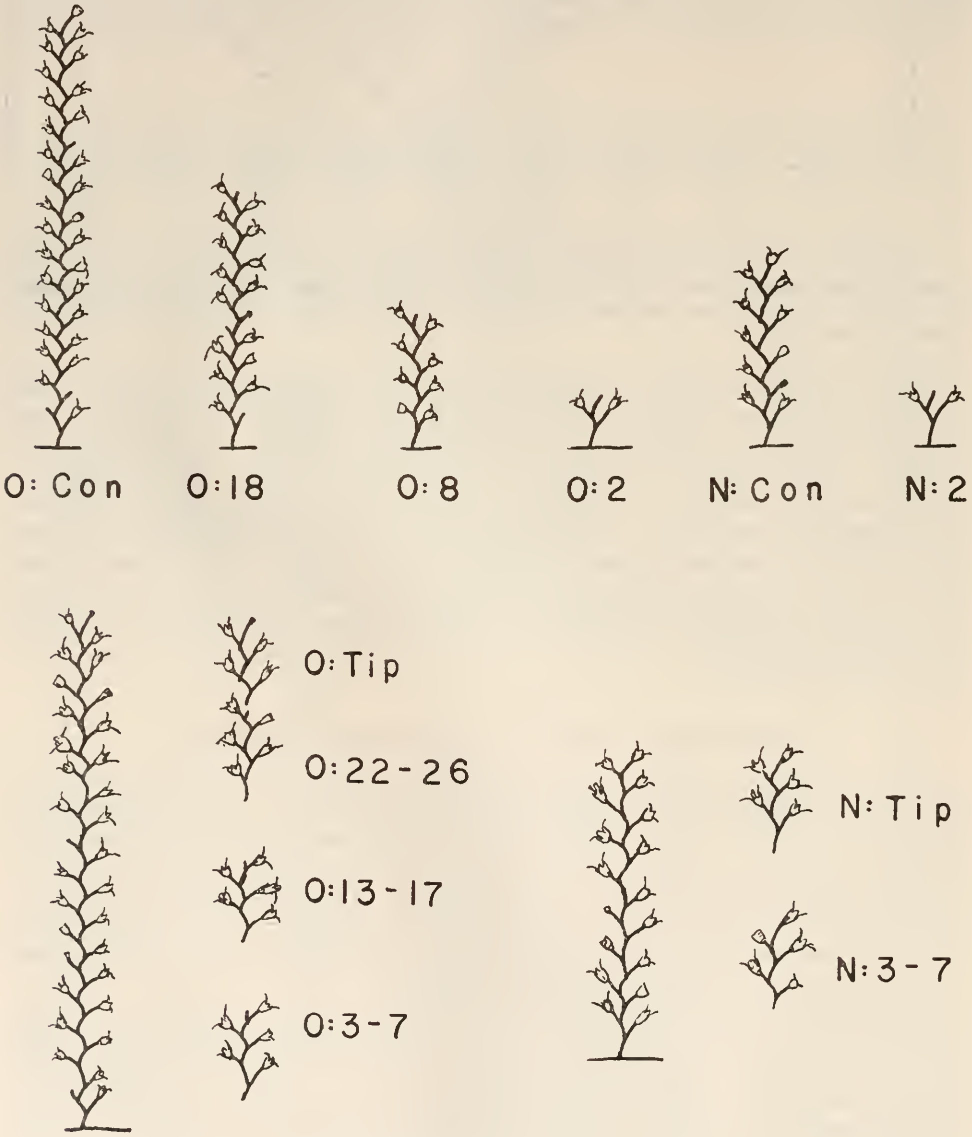

FIGURE 3. Diagrams to illustrate the plan of the experiments as outlined in Table IV.

Table IV

Plan of experiment, shown also in Figure 3. The abbreviations at the left are the designations given the experimental groups

From older series of uprights, 0:

0:con	Controls with about 30 positions, connected with rest of colony
0:18	Trimmed to leave 18 positions, connected with rest of colony
0:8	Trimmed to leave 8 positions, connected with rest of colony
0:2	Trimmed to leave 2 positions, connected with rest of colony
0:3–7	Sections consisting of positions 3 to 7
0:13–17	Sections consisting of positions 13 to 17
0:22–26	Sections consisting of positions 22 to 26
0:tip	Sections with 5 positions including the uninjured terminal position (approximately 32–35)

From younger series of uprights, N:

N:con	Controls with about 10 positions, connected with rest of colony
N:2	Trimmed to leave 2 positions, connected with rest of colony
N:3–7	Sections consisting of positions 3–7
N:tip	Sections with 5 positions including the uninjured terminal position (approximately 12–15)

Since the development of a hydranth involves more differentiation and organization than the production of a free stolon, we think of the former as more "difficult" as well as more effective in respect to the normal growth of the colony.

Both in describing the results and in the discussion the word *significantly* has been used only when differences are significant at the 1% level as determined by "t" test.

The data are presented in Table V, A. The young controls, N:con, have a value of 26.6 hours for positions 12–15. This is significantly faster than the rate for the old controls at 30.7 hours. The old uprights cut near the base, 0:2 and 0:8, have rates well above 40 hours. They are significantly slower than old and young controls and also slower than the young uprights, N:2, similarly amputated. The poorest rate is seen for the few uprights which produced hydranths when cut at the nineteenth internode, 0:18: 56.4 hours (significantly slower at the 5% level than 0:2, in spite of the small number of cases).

The times for the development of the first hydranth are shown in Table V, A in a separate column. Since once development starts, its rate is the same for all groups, differences among them must be a reflection of the time required for healing and preparation for proliferation. The O: – groups are nearly identical one with another but slower than the younger N:2 group.

Table V, B shows the percentages of cases in which the trimmed uprights produced hydranths rather than free stolons at the cut surface. In one column is shown the percentage of cases in which hydranths were produced at the first opportunity, in the last column the percentage of cases which finally produced a hydranth after one or more removals of the stolon which had grown at first. The groups stand in the same relationship one to another whichever column is used. Comparison of the values among the groups in Table V, B with those of Table V, A shows that the uprights which produce hydranths most rapidly are the same ones which more often produce hydranths rather than free stolons.

In the "effectiveness" or "efficiency" of terminal growth the experimental groups stand in the following order: Tips of unamputated young uprights — N : con; tips of unamputated old uprights — O : con; young uprights amputated near their base — N:2; old uprights amputated at the third or ninth internode — 0:2 and 0:8; and old uprights amputated at the nineteenth internode — 0:18.

TABLE V

The development of hydranths and free stolons from amputated uprights

Experimental group	Position of new hydranths	Hours for hydranth production			Hydranths produced instead of free stolons		
		Number of cases	Cone to cone	Cut to cone	Number of cases	% the first time	% at any time
Trimmed Uprights							
		A			B		
0:2	3	14		37.9 ± 6.0	31	16	74
	4–6	38	43.9 ± 12.2				
0:8	9	9		35.9 ± 4.9	16	19	75
	10–12	25	47.6 ± 12.8				
0:18	19	7		37.1 ± 5.4	12	8	58
	20–22	5	56.4 ± 20.3				
0:con	32–35	39	30.7 ± 4.2				
N:2	3	26		32.5 ± 2.3	33	52	97
	4–6	69	35.2 ± 8.1				
N:con	12–15	71	26.6 ± 4.0				
Isolated Sections							
		C			D		
0:3–7	8	9		29.3 ± 7.1	19	16	32
	9–11	21	43.9 ± 12.4				
0:13–17	18	4		33.8 ± 7.1	15	13	27
	19–21	11	44.1 ± 10.1				
0:22–26	27	9		30.2 ± 4.2	16	56	68
	28–30	26	36.2 ± 6.1				
0:tip	32–35	45	35.6 ± 8.3				
N:3–7	8	17		32.4 ± 7.1	19	74	95
	9–11	48	43.0 ± 12.4				
N:tip	12–15	60	36.0 ± 9.2				

Before considering the results of the measurements on the isolated sections of uprights, it is necessary to point out that the values for them cannot be directly compared with those of the specimens still attached to the rest of the colony. Because there was a good deal of regression of hydranths in segments isolated from lower levels of the uprights and because it seemed essential to be able to compare the group one with another, we fed each specimen two brine shrimp a day for each position present (whether a hydranth was at each position or not). This gives as constant a nutritional intake as can be achieved. It is at a level known to be adequate for moderate, but not maximal, growth (Crowell, 1957).

As before, the times are calculated separately for the first hydranth to be produced (from the time of cutting) and for the subsequent three. Data are presented in Table V, C and D.

The time required for the development of the first hydranth is nearly equal for the four groups.

The growing tips of new and old colonies are almost identical in rate although they are at levels 20 positions apart. That their rates are somewhat slower than those of intact uprights is interpreted as due to the lower quantity of food received (discussed above). Levels farther from the growing tips have slower rates. The level nearest the tip, 0 : 22–26, is essentially equal to the tip, and significantly better than the lower two levels, 0 : 13–17 and 0 : 3–7 which are alike. N : 3–7, even though it came from a young upright, and was only about 7 positions below the growing tip, is also slow.

In the case of the amputated uprights it will be recalled that there was a close parallelism between rate of hydranth production and the tendency to produce hydranths rather than free stolons. The same is true for the isolated sections (Table V, D), with one exception: the N : 3–7 group had a slow rate but rarely produced stolons.

Discussion and Conclusions

The continuous record for 60 days of the terminal growth of uprights (Fig. 2) shows that when nutrition is optimal, the growth rate is maximal for about 10 days, then gradually reaches a new and lower level which it maintains thereafter. One can postulate that whatever factors reduce the rate from about 24 to 33 hours, these have no further effect, and, at the somewhat slower rate, terminal growth could continue forever. In nature a long stem would, of course, eventually be broken off. In the laboratory one could cut off the distal portion and follow its history, an experiment which we have not yet carried out. The "immortal" hydras described by Brien and Reniers-DeCoen (1949), and the successful indefinite asexual reproduction of some oligochaetes and turbellarians (*e.g. Stenostomum*, Sonneborn, 1930) are examples of growth without limitation somewhat comparable to the situation described here.

Earlier experiments (Crowell, 1957) have shown that the height or age of an upright determines quite precisely the extent to which lowered nutritive level affects the rate of terminal growth. This effect of age (or height) is evident in stems when they are only a few positions high or a few days old and indicates that at least one factor which can influence growth is accumulating long before it can become effective in well-fed colonies.

The experiments involving the trimming of uprights and isolation of sections at different levels were designed with some prejudice in favor of a correlation between the rate of terminal growth and the age of the tissues. Although the terminal growth zone is always young in actively growing colonies, it is still possible that there occurs an inherent slowing down with time, independent of factors external to the terminal growth region itself. In the absence of this condition it may be possible that the older tissues below the terminal zone, as they become older, are the source of factors which adversely affect terminal growth.

Analysis of the data has made it necessary to consider four possibilities. 1) Correlation with age of tissues as discussed above. 2) Inhibitory substances arising from hydranths already present, but not correlated with the age itself of these hydranths—perhaps a process of like inhibiting like. 3) Deficiency of nutrition as the distance from the stolon becomes greater. This might merely be a consequence of less efficient hydroplasmic streaming in the distal portion of tall uprights. 4) Any combination of the above.

The factors suggested are not the only possible ones. Hydranth regression, production of lateral branches and the development of gonangia may have effects, but this question has not yet been examined. There are, of course, still other possibilities.

If slowing of terminal growth were inherent to the growing terminal portion of the upright, one would not expect this to be expressed during only the production of the tenth to twentieth positions. Tips of old and young uprights, different in length by 20 positions, when isolated showed the same rates of terminal growth (0 : tip and N : tip in Table V, C).

When we compare groups 0 : 3–7, 0 : 13–17, 0 : 22–26, and 0 : tip, the similarity of rate between the latter two suggests that no marked slowing has occurred 7 positions below the tip. However, those about 15 positions below the tip, 0 : 13–17, show a significantly slower rate. The oldest group, 0 : 3–7, is the same as 0 : 13–17. It should be emphasized that each of these specimens received the same amount of food; hence differences cannot be accounted for on the basis of nutritional intake. Further evidence of a correlation with age is seen in the comparison of groups N : 3–7 and N : tip. The former, comprising tissue about 7 positions back from the tip, shows a significantly slower rate than the tip. Comparisons in Table V, A between N : 2 and N : con and between N : 2 and 0 : 2 also show a correspondence between rate and age of tissues.

It is evident that there is no aging at the growing tip itself. It is also clear that the rate of hydranth formation at increasing distances from the tip and in older tissues is slower. The tissues do not express the consequence of aging until about 7 hydranths have been produced beyond them, that is, about 10 days after the tissue had been first established. At this time this effect increases sharply; soon it becomes maximal and thereafter there is no further decrease in rate related to the increasing age of the tissues. The similar rates shown by 0 : 3–7 and 0 : 13–17 support this last statement.

Not all of the results are consistent with the idea that aging effects explain the slowing of terminal growth. The experiments do not distinguish between the role of possible inhibitors and that of the efficiency of circulation. With increasing length of upright there is not only an increase in the number of hydranths to produce an inhibition, but also a greater length of coenosarc separating the proliferating zone from the basal stolon. The chief indication that one or both of these factors are operating is seen in Table V, A, particularly for the old uprights. With increasing distance from the stolon (decreasing age of tissues) there is an increase in the time required for hydranth production. Statistically, 0:2 with the faster rate is not significantly different from 0:8, but is different at the 5% level from 0:18. These results are the opposite to those expected as a consequence of aging and indicate that some factor(s) exert an effect opposite to that of aging.

The frequency with which the cut specimens developed free stolons was higher the slower the rate of hydranth production. To this generalization there was one exception: the N:3–7 group which produced relatively few free stolons but had a slow rate for hydranth production. This same group has been mentioned earlier in connection with the effect of age of the tissues. It had a slower rate than tissue of about the same age belonging to older uprights, the 0:22–26 group. We are unable to decide whether we should regard this case as anomalous in respect to its rate or in respect to the frequency of free stolon production. Its significance may lie in the hint which it gives that the factors which influence the production of free stolons may be different from those which control rate.

One result, so far mentioned only incidentally, is shown in Table V, A and C. For those cases in which the distal tip had been severed, the rate of replacement of the first hydranth, that is the one arising from the cut surface, is tabulated separately from the rates for successive hydranths. For all of the groups there is little difference in the rates at the cut surface but marked differences for hydranths subsequently produced. Lund (1923) found differences in replacement of hydranths of *Obelia* correlated with the distance from the growing tip. The absence of such differences in our specimens is unexpected.

Although the rates for the production of the hydranth from the cut surface are similar for all our cases there was great variation in the frequency of free stolon production by these same cut ends. Apparently if a hydranth is to develop, it can begin to do so with about the same speed regardless of the level of the cut or age of tissue. A correlation with age is seen in the frequency of free stolon production, but the effect of age applies to hydranth production only after the first has been produced.

The general conclusion from these many considerations and comparisons is that there is a period of maximal rate of terminal growth expressed by uprights only during their first 10 days; after this the zones of prospective terminal growth become adversely affected by factors external to themselves. Although the age of cut sections of stems is one factor correlated with slower hydranth production and with the development of free stolons instead of hydranths, many of the results cannot be explained on this basis. What these other factors may be can only be conjectured until further experiments are carried out.

SUMMARY

1. When terminal growth rate was measured for stems of well-fed colonies of the hydroid *Campanularia flexuosa* for a period of 60 days, it was found that this rate is constant for about ten days, becomes progressively slower for the next 10 to 15 days, and then remains constant.

2. Isolated sections of stems of different ages differ one from another both in

respect to the time required for the production of additional terminal hydranths, and in their ability to produce hydranths rather than free stolons. In general the same experimental groups which most readily produce hydranths following cutting produce them at fairly high rates.

3. Older levels of stems are in general less efficient in the rate of terminal hydranth production and in the ability to produce hydranths rather than free stolons. Not all of the results can be explained on the basis of an effect of aging. The possible role of inhibitors and of differences in efficiency of circulation must be considered.

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RESPIRATORY METABOLISM OF THE FIDDLER CRAB UCA PUGILATOR FROM TWO DIFFERENT LATITUDINAL POPULATIONS

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By definition a poikilotherm is an animal whose internal state fluctuates with temperature changes in its environment, a lowering of environmental temperature resulting in a reduction of metabolism or activity. Accordingly, it is to be expected that a poikilotherm living in colder habitats and subjected seasonally to low temperatures would have a lower metabolic rate than would the same species inhabiting a region of higher temperatures. Nevertheless, considerable information indicates that many cold-blooded animals living in colder habitats operate at higher rates than those we could expect by deduction from their rate-temperature curve. This is supported by the observation that many species of marine invertebrates are as active in colder seas as those in warmer waters. This phenomenon of compensatory adaptation of rate of metabolism or activity has been recently reviewed by Bullock (1955) and Prosser (1955).

Compensatory adaptation has been studied in three different ways: (1) by experimental acclimation of animals at various temperatures; (2) by observation of animals at various seasons; and (3) by comparisons of groups of individuals varying in latitude.

It has been observed, especially among the aquatic poikilotherms, that certain species from the northern and southern regions may show rather similar rates of functions when measured at their own habitat temperature. Many animals from higher latitudes tend to show higher rates than animals from lower latitudes, when measured at any given temperature.

With respect to latitudinal temperature compensation, we find numerous investigations. Different species of a genus or different individuals of a given species show about the same rate functions in Greenland, the North Sea and the Mediterranean (Sparck, 1936; Thorson, 1936; Wingfield, 1939). Thorson (1956) says (p. 695): ". . . an arctic *Macoma* community at 0° C. shows, roughly, a similar metabolic rate, a similar rate of growth, and similar feeding habits, as a boreal *Macoma* community at 8° C., or a Mediteranean community at about 12° C., or a tropical community at a still higher temperature."

The more recent investigations deal, in large part, with studies of the same species from different latitudes, and the results obtained by Roberts (1952) and Rao (1953) on crabs and mussels along the west coast of the United States are also in favor of a certain adaptation of metabolism or activity to temperature in different populations.

In 1948, Dr. Dorothy Bliss (personal communication) undertook a few experiments concerning the tolerance to low temperatures of two species of *Uca* from Woods Hole, Massachusetts, namely *U. pugnax* and *U. pugilator,* and of two latitudinal populations of *Uca pugilator* from Woods Hole and from Florida. These experiments suggested first, a very obvious difference in the resistance to cold between the two species of *Uca* from Woods Hole, and secondly, a noticeable, although less impressive, difference in the behavior of the northern and southern populations of *Uca pugilator* when very low temperatures were reached, that is to say, between 1° and 3° C.

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The purpose of this present investigation was to continue with this problem of physiological variations and compensatory mechanisms, perhaps resulting from the different latitudes at which Woods Hole and Florida crabs are living. *Uca pugilator* (Bosc), the sand fiddler, according to Rathbun (1918) and Crane (1943), ranges from Boston Harbor to Galveston, Texas. Up to now, a single species has been described, and Jocelyn Crane (personal communication) states that the taxonomic problem of Woods Hole and Florida *Uca pugilator* has not yet been investigated.

We have been concerned, first, with comparisons at a given temperature of the metabolism of these animals, measured as oxygen uptake, and, in addition, with observations of their activity and their resistance to cold.

This work has been carried out during a sojourn at Harvard University while holding a Smith-Mundt Fulbright travel grant. It was supported, in part, by Research Grant B–623 from the National Institute of Neurological Diseases and Blindness, Public Health Service. I am happy to express my thanks to Dr. J. H. Welsh for his kind hospitality, for his constant and valuable support and for all material facilities he provided. I am also much indebted to Dr. Dorothy Bliss who helped me with the use of the respirometers and showed a constant interest in the course of my work.

Materials and Methods

Crabs from Woods Hole (latitude 41° N) and from Florida (vicinity of Englewood, latitude 28° N) were shipped periodically to Cambridge, Massachusetts. As soon as they arrived, they were placed in containers with a small amount of sea water and kept at room temperature, that is to say, at an average of 20° C. They were fed fish twice a week. Water was changed regularly. Some experiments dealt with animals which were kept for various lengths of time at 10° C. Throughout the work, only male crabs were used.

For the determinations of the rates of oxygen consumption, Dixon volumetric respirometers were employed. Carbon dioxide was absorbed either by Ascarite or by 20% KOH. In some experiments, in order to absorb any excreted ammonia, a cupric chloride solution was added in the apparatus containing KOH. No significant differences in readings were noted. Two temperature baths were used in measuring oxygen consumption; one was maintained at 15° C., the other at 1.4° C.

A period of time varying between one-half hour and three-fourths of an hour was devoted to equilibration of the apparatus. Every experiment was run at the same time of the day (between one P. M. and six P. M.) to avoid fluctuations due to the diurnal variations in metabolism. Readings were made at convenient inter-

TABLE I

Respiratory rates of Uca pugilator measured at 1.4° C.

Weeks spent at 20° C.*	Woods Hole						Florida						Significances of differences between Q_{O_2}	
	Size of sample	Wet body weight (g.)		Q_{O_2} (mm³/g./hr.)			Size of sample	Wet body weight (g.)		Q_{O_2} (mm³/g./hr.)			t	P
		range	mean	range	mean	standard deviation		range	mean	range	mean	standard deviation		
1							12	2.11–4.42	2.96	2.1–7.9	4.1	1.60		
2	7	2.57–3.80	3.10	2.5– 9.9	8.1	2.46	11	2.10–3.85	3.19	2.2–8.6	4.8	1.92	2.99	<0.01 highly signif.
3	10	2.85–4.00	3.39	1.3–13.3	6.0	3.86	9	2.25–3.40	2.85	2.2–8.1	4.2	1.69	1.71	0.10 no signif.
4	8	2.48–3.52	2.86	2.1–11.4	7.0	2.60	10	1.83–3.74	2.39	2.1–8.7	4.3	1.82	2.12	<0.001 highly signif.
6	8	2.80–3.32	3.22	2.7–11.0	5.9	2.81	11	2.45–3.93	3.06	1.5–5.8	3.9	1.10	2.11	<0.05 signif.
7	7	2.00–3.25	2.70	6.0–10.2	8.0	1.62	10	2.25–3.35	2.83	1.9–8.1	4.8	2.17	2.14	<0.01 highly signif.
8							8	1.05–4.43	2.45	3.1–6.4	4.3	1.15		

* No observations made during fifth week.

vals. The first hour's readings were discarded, for we observed an excessive oxygen consumption at 1.4° C. and at 15° C. After one hour, more uniform uptake occurred. (Recently, Grainger (1956) has observed a similar phenomenon with *Artemia.*) Readings were recorded during the following three hours. Results have been corrected to standard temperature and pressure.

The observations on cold tolerance were made on the animals after they had been taken out of the respirometers.

Results

I. *Rate of oxygen consumption as a function of latitude*

1. *Measurement at 1.4° C.*

According to the previously mentioned experiments of Dr. Bliss, we chose the temperature of 1.4° C. for our comparisons between the rates of oxygen consumption of Woods Hole and Florida *Uca.*

The measurements were begun on the first day after arrival for southern crabs and on the tenth for the northern populations. Starting on September twenty-seventh, they were continued for an eight-week period for Florida crabs and a seven-week period for those from Woods Hole. The stocks of crabs of both regions were kept at room temperature (20° C.). Each day the rates of oxygen consumption of two crabs of each population were measured and then the animals were discarded. In no case was an animal used twice.

a) The results, as seen in Table I, indicate that there is an obvious difference between the rates of oxygen consumption of the two populations when recorded at this temperature and converted to standard conditions. We tested the significance of the differences between these rates in using the t test of significance after

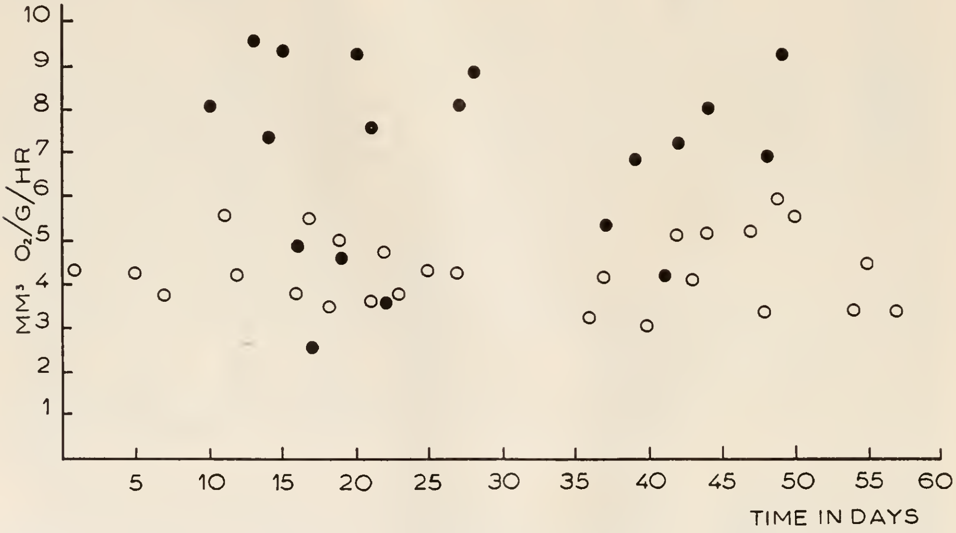

FIGURE 1. Oxygen consumption of *Uca pugilator* from Florida (○) and from Woods Hole (●), as a function of time spent at 20° C. Measurements were made at 1.4° C.

TABLE II

Respiratory rates of Uca pugilator measured at 15° C.

Populations from:	Size of sample	Wet body weight (g.)		Q_{O_2} (mm³/g./hr.)			Significance of difference between Q_{O_2}	
		range	arithm. mean	range	arithm. mean	standard deviation		
Woods Hole	12	2.32–3.85	2.33	29.3–16.8	25.0	4.60	$t=1.15$	$P>0.10$ no signif.
Florida	14	1.30–3.17	3.15	37.3–16.0	22.2	6.90		

Chambers (1952). This indicates clearly the possesion of a compensatory mechanism by *Uca pugilator* in relation to its habitat temperature, the northern group showing the higher rate.

b) During their sojourn at a common temperature of 20° C. no significant change in the rate of oxygen consumption could be noticed either in Florida *Uca* or in Woods Hole *Uca*. A straight horizontal line could represent the steady rate of oxygen consumed over the period of seven or eight weeks (Fig. 1). Therefore, the same amplitude of divergence still exists between the two groups at the end of their sojourn at 20° C.

c) A comparison between the two series of values obtained shows that there is a greater spread in the values of Q_{O_2} for Woods Hole animals than for those from Florida.

2. *Measurements at 15° C.*

Determinations of the resting metabolism of the two populations made at the common higher temperature of 15° C. gave a mean value of 25 mm³/g./hr. for Woods Hole *Uca pugilator* and a mean value of 22.2 mm³/g./hr. for the Florida group. The difference is not statistically significant (Table II).

3. *Temperature coefficient*

It would be reasonable to expect to find an alteration of the sensitivity of metabolism to temperature changes among the cold-adapted population from Woods Hole. This would mean that the northern animals could withstand large temperature variations and show less change in their oxygen consumption than those living in the south. In fact, such findings have been revealed by different investigators for various physiological activities in many different species (Rao, 1953; Dehnel, 1955; Tashian, 1956). Rao and Bullock (1954), in their review of the relation of Q_{10} to the temperature at which the animal is adapted, give good evidence of a decrease in Q_{10} with high latitude, in spite of contrary results reported by Scholander *et al.* (1953).

From our metabolism studies on *Uca pugilator* from Woods Hole, we find, between 1.4° C. and 15° C., a Q_{10} of about 2.47. If we compare the rates of Florida *Uca* at the same temperatures, we get a Q_{10} of 3.52. This shows a decrease in Q_{10}, with increasing latitude. Recently, Tashian (1956) found a Q_{10} of 2.6 for *Uca speciosa* (weight 0.3–1.4 g.) from Key Biscayne, Florida, for temperatures between 14.8 and 24.5° C. If we calculate the Q_{10} from his results on *Uca pugnax,* which were about the same weight as our *Uca pugilator* (3 g.), we find that it is about 3.0 for Florida *U. pugnax* and 1.3 for New York *U. pugnax*. But the comparison of his results with those of the present writer is not entirely valid because of the different choice of temperature range for determination of the rates (see Rao and Bullock, 1954).

II. *Activity*

During the experiments on oxygen consumption, we noticed a difference between the two populations of *Uca pugilator* in their ability to withstand low temperature. After a five hours' stay at 1.4° C., specimens of *Uca* from Florida were entirely relaxed and motionless as though dead. Not a single sensitive region of the body responded to touch. As they recovered, most of them were extremely spastic with occasional movements of their legs. This condition lasted as long as six days. Some appeared as if they were shivering. In October, of forty-two Florida crabs subjected to this temperature, eighteen were found dead on the next day.

Individuals of Woods Hole *Uca* appeared much more resistant under the same conditions and behaved in a different way. Immediately after removal from their stay at 1.4° C., they were also very quiet, but one could notice a few very sluggish movements of their legs, and generally the eyes responded to touch. They recovered rapidly, and sometimes some of them were moving around a quarter of an hour after they had been returned to room temperature. No spasticity at all could be observed. Of forty-two crabs subjected in October to 1.4° C. for five hours, only two were found dead on the next day.

No difference at all could be found between representatives of the two populations after they had been kept at 15° C. for six hours.

III. *Experimental acclimation at low temperature*

Oxygen consumption measurements were also undertaken at 1.4° C., after the animals of the two regions had been kept at a common lower temperature of 10° C., to determine if there was any adaptation of metabolism and activity of the animals at such a low temperature.

Florida *Uca* showed a slight decrease in oxygen consumption for the first seven days of the experiment (in comparison with the measurements made with crabs maintained at room temperature). By the end of the first week, a slight increase in the amount of oxygen uptake was noticed, but may not be really significant. Nevertheless, at this time, a change in the behavior of the Florida crabs was noticeable. Crabs transferred from room temperature to 10° C. became quickly inactive. Only when strongly disturbed did they respond by very slow and sluggish movements. They remained in the water all of the time. This behavior could be observed for five days, after which they gradually began to move, and on the seventh day all of them were found standing up on their legs. Now, much more sensitive, they were disturbed even by a threat of catching them, and many moved out of the water. This recovery of activity, clearly a sign of a certain amount of acclimation, coincided with the observed slight increase in respiration.

It was also observed that after this previous stay at 10° C. the animals were more resistant to a five-hour stay at 1.4° C.; no spasticity was apparent when they became active after exposure at this low temperature. Even after one day at 10° C., they appeared to be able to see and to move their eyes and legs very slowly during their exposure to 1.4° C. After three to eight days at the previous temperature of 10° C., certain individuals recovered within a few minutes after removal from the 1.4° C. bath.

Woods Hole *Uca* kept at 10° C. and compared with animals kept at 20° C. showed an important decrease in their metabolism for the first ten days and then an appreciable increase, but in any case the rate of oxygen consumption was higher than when measured after sojourn at room temperature. After thirty days at 10° C. it decreased again, perhaps because the animals were eating very little at this low temperature.

Discussion

This work is an example to be added to those showing the existence of a latitudinal compensatory mechanism within representatives of a given species. Our results are partially in agreement with those of Roberts (1952), obtained with *Pachygrapsus crassipes*. This author compared the metabolism of four populations differing in latitude along the coast of California. He observed that when the oxygen consumption was measured at a common temperature of 16° C. during the winter, the more northern the populations, the more oxygen they consumed.

They also agree with the latitudinal compensatory differences found in rates of ciliary pumping of water in the mussel *Mytilus californianus* (Rao, 1953). The rates of pumping for unit weight in *Mytilus* of similar weights from Los Angeles, Fort Ross and Friday Harbor are, at any given temperature, much higher for animals from higher latitudes than in those from lower latitudes. However, we must not forget that our own experiments bear upon only a very restricted life span of *Uca pugilator*. Animals of both populations were considered as adult crabs, and their weights did not vary outside the range 1.05 g.–4.73 g. Therefore we could not study the fate of Qo_2 and Q_{10} over an extensive size range but we can satisfactorily and precisely compare these coefficients between our two groups.

The higher rate of metabolism shown by *Uca pugilator* from Woods Hole as compared with Florida individuals was very obvious at the low temperature of 1.4° C. At 15° C. the difference was not significant any longer. This temperature must be very close to the environmental temperature of Woods Hole crabs in October. One might predict that at their own habitat temperature the rates of Florida crabs would be slightly higher than those of Woods Hole crabs. Recently, Tashian (1956) found that in comparing the metabolism of the Florida and New York *Uca pugnax*, at a temperature of 24° C., the rates were quite similar. The measurements having been done during the summer, it happens that 24° C. corresponds to the environmental temperature during the collecting periods. This author deduces that at their normal habitat temperatures the rates of the two populations are similar. If the measurements had been done during the winter when there is a marked difference between the water temperature of the two regions, the results would probably have been different.

After keeping *Uca* for seven or eight weeks at a common temperature of 20° C., we could not, in contrast to Roberts, notice any decrease in the amount of oxygen consumed by the representatives of each locality. The fact that our animals were regularly fed may be responsible for this steady rate of metabolism. Moreover, the difference noticed at the beginning of the experiments between the metabolism of the two populations could not be abolished by a sojourn of eight weeks at a common temperature. Roberts showed that *Pachygrapsus crassipes* from different localities become adjusted to the new temperature of 16° C., at which they have been placed for six weeks, and that the original metabolic differences become doubtfully significant. This is not the case for *Uca pugilator,* at least from Woods Hole and Florida.

From our experiments, it appears as if the two populations of *Uca pugilator* have acquired their own metabolic rates which have become fixed and not susceptible to modification, at least when only one factor of their environment has been changed. The two populations, apparently good examples of "distance isolation," have found conditions of existence quite different at their own habitats, and several ecologic factors are certainly responsible for their differences. According to the quite important difference in latitude, temperature is no doubt the most significant factor.

Whether the physiological differences in metabolism and tolerance to low temperature in *Uca pugilator* of different latitudes are more than phenotypic, we cannot say. Work needs to be done with breeding tests. Knowledge of growth rates, time of sexual maturity, and behavior of the animals in each locality would be very useful.

As we have already noted, no information about differences in morphology of the two populations could be found. After a comparative examination of the individuals, we can say that not very striking morphological characters occur. Nevertheless, after becoming familiar with the animals, we can easily separate the two forms by their color. Woods Hole *Uca,* always darker than those from Florida, are generally bluish-gray. Florida *Uca* are reddish-yellow. The shape of the claw is also slightly different, with longer dactylopodite and propodite for the individuals from Florida.

Thus, the extreme range of *Uca pugilator* does not seem to be accompanied by great morphological modification. In our comparison between *Carcinus maenas* from Atlantic and Mediterranean French coasts, the morphological differentiation appeared more pronounced (Démeusy and Veillet, 1953). But *Uca,* physiologically, is succeeding very well, and it may provide an example of a physiological adaptation previous to important morphological speciation (Crane, 1943).

By their physiological features (rates of oxygen consumption, sensitivity to low temperatures), as well as by a few morphological characteristics, the populations of *Uca pugilator* from Woods Hole and from Florida might be separated into two subspecies.

SUMMARY

Determinations of the respiratory rates of *Uca pugilator* from two different latitudes (Woods Hole and Florida) have been made at 1.4° and 15° C.

1. Woods Hole *Uca pugilator* show a higher rate of metabolism at low temperature than do specimens of the more southerly populations.

2. *Uca pugilator* from the higher latitude are less sensitive to temperature changes than *Uca pugilator* of same weight from a southern latitude. This has been shown by a lower Q_{10} for the Woods Hole population.

3. Woods Hole *Uca* are more resistant to low temperature than Florida *Uca.*

4. The same experiments made after the animals have been left at a common temperature of 20° C., show that a stay of seven or eight weeks under similar conditions does not abolish the metabolic differences observed between populations.

5. These physiological characteristics and some morphological ones might be used to distinguish two subspecies of *Uca pugilator*.

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A QUANTITATIVE EXAMINATION OF TESTICULAR GROWTH IN THE WHITE-CROWNED SPARROW[1]

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Since the pioneer investigations of Rowan (1925, 1926) and Bissonnette (1930) on *Junco hyemalis* and *Sturnus vulgaris,* respectively, there has been accumulated an abundance of experimental evidence which indicates that artificial elongation of the daily photoperiod in winter can cause testicular growth and development in a substantial number of temperate zone species of birds. Much of the available information has been reviewed or cited by Bissonnette (1937), Wolfson (1945, 1952), Benoit (1950), Burger (1949), Hammond (1954), Aschoff (1955) and Schildmacher and Rautenberg (1953). In general these studies contain relatively little information on the actual rates of testicular development as functions of the length of the daily photoperiod, although the investigations of Burger (1948, 1953) on *S. vulgaris,* Bartholomew (1949) on *Passer domesticus,* and Winn (1950) on *J. hyemalis* are exceptions in this respect. Despite the sparsity of data on rates of testicular development the investigations of the photoperiodic stimulation of testicular growth and development have provided the basis for widely accepted theories in which the increasing vernal day-length is regarded as the primary stimulator and timer in the testicular cycle. These theories are supported by the observations that, in at least most species studied, retention at winter day-lengths results in no development or relatively slight development. Consequently an appraisal of the photoperiodic theories indicates them to be qualitatively rational and logical, at least for many temperate zone species, but deficient with respect to quantification. Because of this deficiency it is not possible to evaluate, either individually or collectively, the roles of other variables in the natural course of testicular development. It is the function of this paper to present approximate quantifications of the function of day-length in testicular development and consequently to indicate the approximate magnitude of the roles of other variables in natural testicular development in a population of the white-crowned sparrow, *Zonotrichia leucophrys gambelii.* The analyses to be presented here suggest arguments against the objections of Blanchard (1941) and Marshall (1952, 1955) to the photoperiodic theories. The data on which our analyses are based were obtained in the course of an extensive series of experiments on the mechanism of photostimulation of testicular development.

MATERIALS AND METHODS

Our experimental birds were captured with Japanese mist nets from a wintering population in the Snake River Canyon in southeastern Washington (Mewaldt and

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[1] The data on which this paper is based were obtained in investigations supported by the Office of Naval Research (Contract Nonr-1520) and by funds made available for biological and medical research through State of Washington Initiative Measure No. 171.

Farner, 1953). Prior to the beginning of the experiments they were held in large outdoor aviaries. For experimental lighting they were placed, one or two per cage in small cages (8½ × 10 × 16 inches), or three or four per cage in larger cages (12 × 24 × 18 inches). Illumination was provided with incandescent lamps at an intensity of 40–60 foot candles which is substantially above the maximal intensity (*i.e.,* minimum intensity at which maximum rate of development occurs) of about four foot candles. The birds were fed a vitamin- and mineral-enriched chick-starter mash prepared according to a formula developed by the Department of Poultry Science of the State College of Washington. Food and water were available at all times. Except as otherwise noted the experimental birds were held at 20–24° C.

Immediately after removal, testes were placed in acetic acid-formaldehyde-ethanol fixing mixture. After five days they were transferred to 70% ethanol and five days thereafter weighed with a Roller-Smith torsion balance.

We are indebted to Drs. Richard A. Parker and Morris S. Knebelman for suggestions and criticisms concerning the mathematical analyses and interpretation of the data. Drs. I. O. Buss and Albert Wolfson have made criticisms and suggestions concerning the manuscript. Some of the data are from experiments conducted by Dr. L. R. Mewaldt while he was a member of our research group.

TESTICULAR WEIGHT AS A FUNCTION OF TIME WITH A CONSTANT DAILY PHOTOPERIOD

An examination of the weights of the testes taken at intervals during a period of treatment with constant daily photoperiods of stimulatory duration suggests a relationship which approximates a logarithmic growth curve until the combined testicular weights reach about 200 mg. If this is a good approximation then the relationship between time and testicular weight may be expressed as

$$\log_{10} W_t = \log_{10} W_o + kt, \tag{1}$$

where W_o is the initial testicular weight in milligrams, W_t is the weight at t days, and t is the period of treatment in days. As an illustration and test of this relationship, data from three experiments have been combined in Figures 1 (first-year birds) and 2 (adults). These three experiments involved treatments with 15-hour daily photoperiods at 0° C. mean temperature, 20-hour photoperiods at 20° C., and 15-hour photoperiods at 22° C., respectively. Since the rate constants (k), with t expressed in days, are different, t in Figures 1 and 2 is expressed in arbitrary relative units (1 = time required to attain 100 mg. combined testicular weight). On this basis there were no apparent differences among the three groups; therefore, they are not differentiated in Figures 1 and 2. An inspection of Figure 1 indicates that, for first-year birds, there is a reasonably good linear relationship between the logarithms of testicular weight and time up to about 200 mg. ($\log_{10} =$ 2.3) and hence a reasonably good conformance with equation (1). For adults (Fig. 2) it appears possible that a weak S-relationship may exist between t and log W_t. However, for purposes of comparison of rates of development among experimental groups, it appears that no useful purpose can be effected in seeking

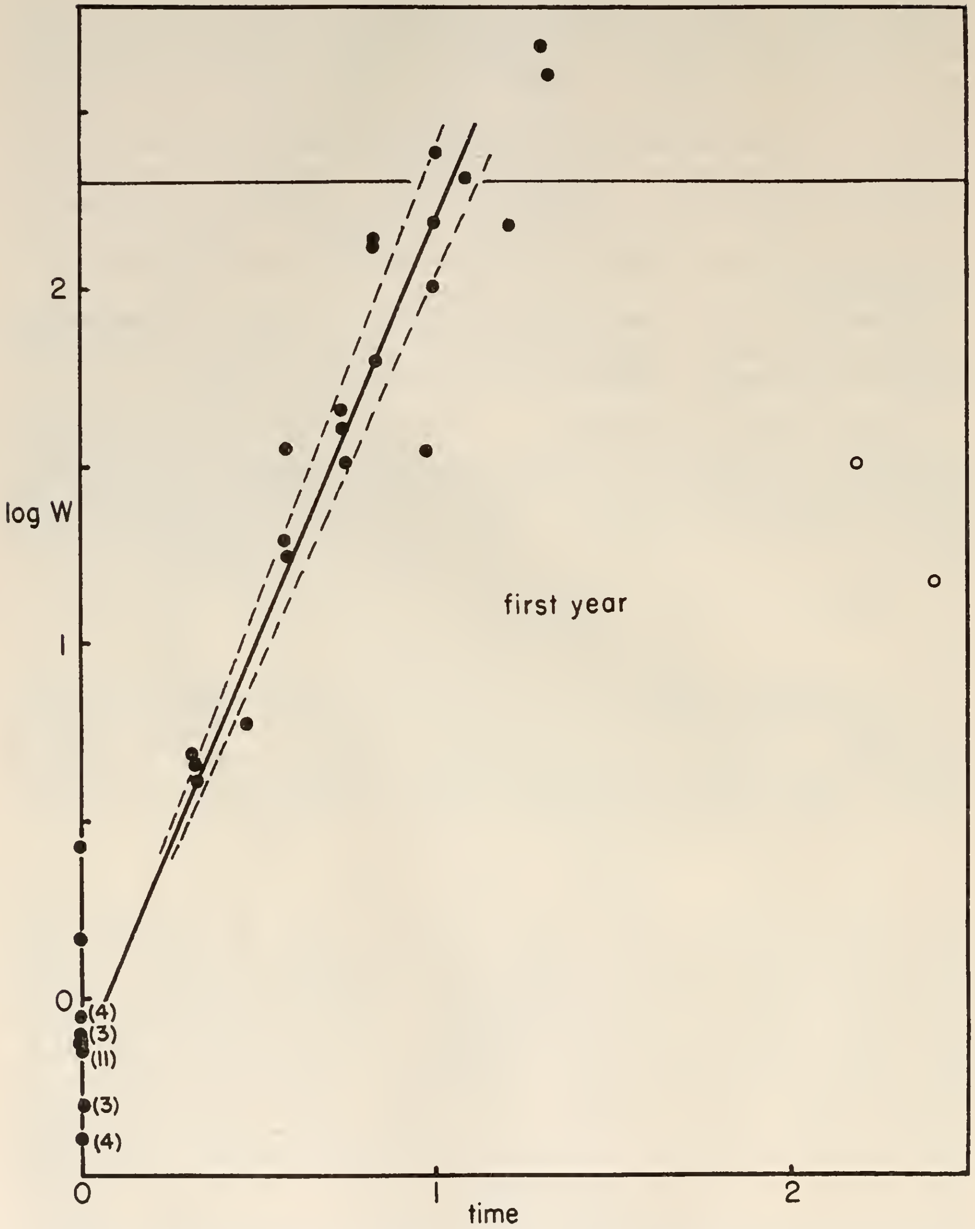

Figure 1. Combined testicular weights (*W*) of first-year white-crowned sparrows as a function of time with constant daily photoperiods of stimulatory duration. Time in arbitrary units (see text). Closed circles represent developing testes; open circles represent regressing testes. Broken lines represent upper and lower 95%-fiducial limits of the slope.

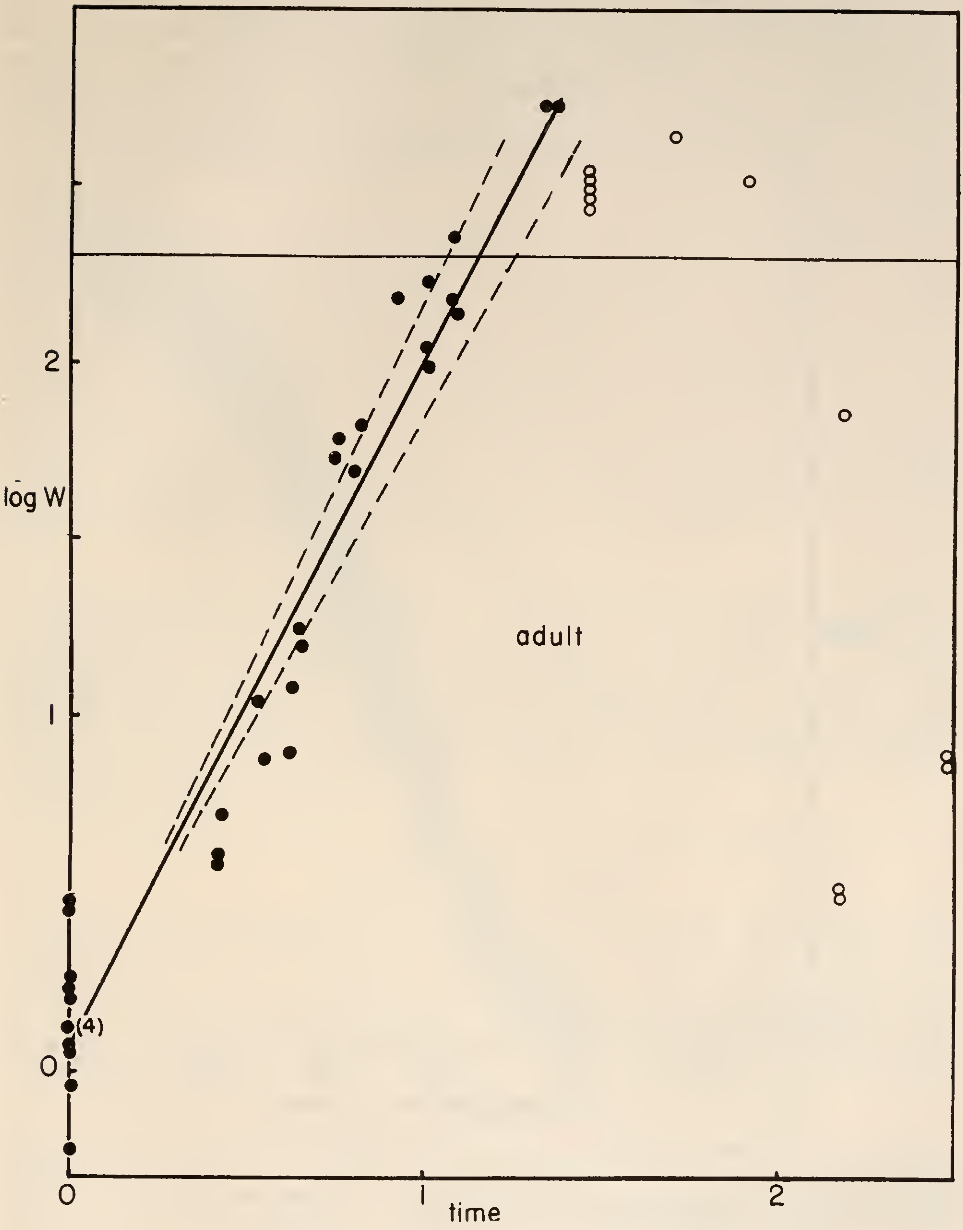

FIGURE 2. Combined testicular weights (W) of adult white-crowned sparrows as a function of time with constant daily photoperiods of stimulatory duration. Time in arbitrary units (see text). Closed circles represent developing testes; open circles represent regressing testes. Broken lines represent upper and lower 95%-fiducial limits of the slope.

a more precise relationship. Because of the less precise relationship between t and W_t for adult birds, it is now our policy to perform all critical experiments with first-year birds. The solid lines in Figure 1 and 2 are drawn according to the values of k obtained by the procedure outlined by Mood (1950, Chapter 13); similarly the broken lines represent the upper and lower 95% fiducial limits, also obtained according to Mood. In order to minimize the effect of possible non-linear relationships between log W_t and t it is now a practice in our laboratory to kill birds when the testes have attained a combined weight of about 100 mg.

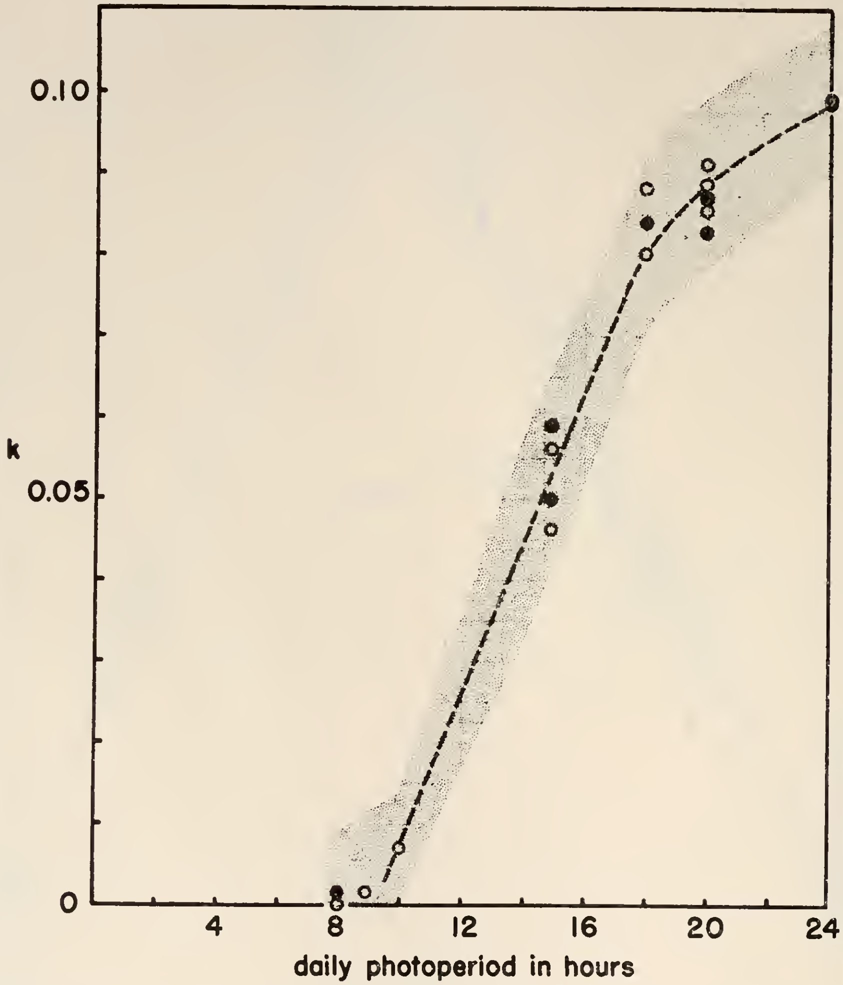

FIGURE 3. The rate of testicular development as a function of the duration of the daily photoperiod (p). See text for definition of rate constant (k). The shaded area encloses the upper and lower 95%-fiducial for all points. Open circles represent samples of first-year birds; closed circles represent adjusted means for samples of adults.

THE TESTICULAR GROWTH RATE CONSTANT (k) AS A FUNCTION OF THE LENGTH OF THE CONSTANT DAILY PHOTOPERIOD

An examination of k as a function of the length of a constant daily photoperiod (p) has been effected by analysis of 18 series of data (11 first-year and 7 adult) for photoperiods varying in length from 8 to 24 hours. For each of these an estimation of k and the approximate 95% fiducial limits for k were obtained according to Mood (1950). These are given in Figure 3. In the calculation of the 95% fiducial limits for k the same W_0 sample was used for all groups with the same W_0 date; the fiducial limits thus obtained are therefore to be regarded as estimated limits rather than true fiducial limits. As indicated above, k is greater for first-year birds than for adults, approximately 1.16 times as great for the series of data used here. Therefore in plotting the data in Figure 3, the adult values, both for estimates of k and the approximate fiducial limits, were multiplied by 1.16. The broken line, suggesting a functional relationship between p and k, has been drawn by inspection. The shaded zone on either side encloses all of the estimated fiducial limits for the individual estimates of k. It must be emphasized that this shaded zone does not represent the fiducial limits for the slope of the curve. The linear portion of the curve (between 10 and 18 hours) may be represented by

$$k_{\text{1st-yr.}} = 0.009(p - 9.1). \tag{2}$$

Obviously there is an uncertainty in the nature of the functional relationship between p and k for values of p less than 10 hours. The relatively small changes in W with respect to the natural variability of W and the errors in measurement involved with small values of W make this a difficult problem. Fortunately, as it will become evident subsequently, the values of k in this range are sufficiently small so that the calculations based on equation (2) are not serious even when it is extrapolated linearly to $k = 0$.

THE ROLE OF ENVIRONMENTAL TEMPERATURE IN THE RATE OF TESTICULAR DEVELOPMENT

For *Z. l. gambelii* it has been demonstrated earlier (Farner and Mewaldt, 1952, 1953) that the rate of testicular development with a fixed daily photoperiod of stimulatory duration is a function of environmental temperature between 0° and 22° C. It should be emphasized, however, that elevation of environmental temperature has no effect when the photoperiod is below stimulatory level (Farner and Mewaldt, 1953). Assuming, for purposes of estimation, that the effect of temperature is linear with respect to k, the following relationship should hold:

$$k_B/k_A = 1 + c(T_B - T_A). \tag{3}$$

We have estimated the value of c on the basis of two groups of birds subjected to 15-hour daily photoperiods, one at a mean environmental temperature of 1° C., the other at 22° C. On the basis of mean values of k for these two groups, c has a value of about 0.009 degrees^{-1}. Substitution of this value for c in equation (3) indicates that the role of temperature must be relatively minor. It must be observed here, however, that the responses of the lower temperature group were sufficiently variable so that, were the true k near the lower 95% fiducial limit, the value of *c* would be approximately 0.019. Engels and Jenner (1956) have examined the effect of environmental temperature on the rate of testicular development in *J. hyemalis* subjected to daily photoperiods of 10–12 hours. Although differences in methods and analyses do not allow a direct comparison, it appears that *c* for this species must be of the order of 0.02–0.03. Similarly, from the data of Burger (1948) for *S. vulgaris* we have estimated *c* to be about 0.02. For these three species, then, it appears that the role of environmental temperature in testicular development is of a small, although similar, order of magnitude.

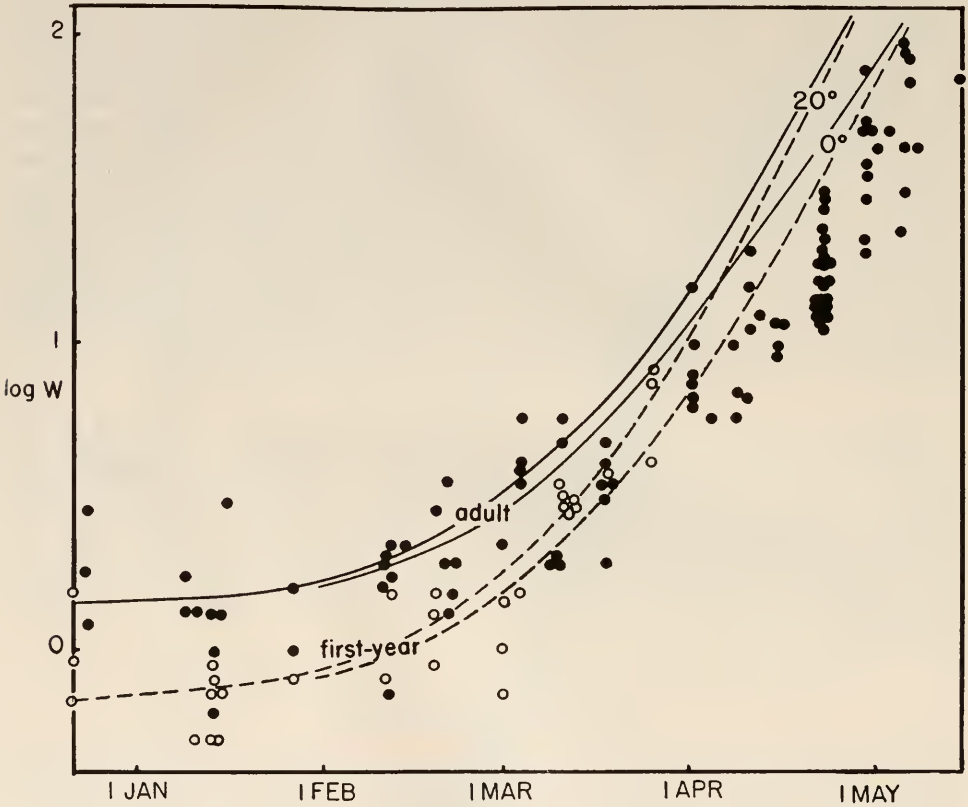

Figure 4. A comparison of predicted curves of testicular development with natural development. Open circles represent combined testicular weights for first-year birds taken from the Snake River Canyon population; closed circles represent combined testicular weights for adults from the same population. These groups cannot be distinguished after the prenuptial molt which occurs in late March and early April. The four curves were calculated according to equation (4) including the adjustment for temperature effects indicated in equation (3).

A Predicted Course of Testicular Development Under Natural Conditions Calculated on the Basis of Laboratory–Established Rates

Using the empirical relationships presented above, an attempt has been made to "predict" the course of vernal testicular development. Since environmental temperature is an irregularly fluctuating variable, two temperature values, 0° C. and 20° C., were used for the construction of two predicted curves. For the most part the environmental temperatures fall within these limits although some subzero nights occur in January and February. The four curves in Figure 4 are based on values of k from Figure 3 assuming that k is a continuous positive function of p between the limits of 0 and 24 hours, k being insignificantly (< 0.002) small for all values of p below 9.1 hours. Calculations were begun with 21 December (day-length about 8.8 hours) when the mean logarithms of the testicular weights for adults and first-year birds were $+0.15 \pm 0.10$ and -0.17 ± 0.16, respectively. As p increases, the correspondingly larger values of k were employed; the operation may be represented by

$$\log_{10} W_n = \log_{10} W_o + \sum_{i=1}^{n} k_i, \tag{4}$$

where n is the number of days after the initial date in the calculation.

If the relationship between p and k assumed here is correct, then the day-lengths following the termination of the refractory period in mid-November (Farner and Mewaldt, 1955) would be stimulatory. Accordingly the curves in Figure 3 have been extrapolated back into November. This extrapolation indicates weights for early November which are within the range of one standard deviation of December weights and well within the range of early November weights.

An alternative interpretation of the functional relationship between p and k is that expressed by equation (2). Four curves (adults at 0° C. and 20° C., first-year birds at 0° C. and 20° C.) were therefore constructed using the equation

$$\log_{10} W_n = \log_{10} W_o + n\bar{k}_{1 \ldots n} \tag{5}$$

in which,

$$\bar{k} = 0.009 \frac{(\sum_{i=1}^{n} p_i)}{n} - 0.082. \tag{6}$$

The curves obtained thus were not detectably different from those obtained by the operation noted in equation (4) and plotted in Figure 4. In the use of equations (4), (5), and (6), p has been assigned the value of the time between sunrise and sunset. Obviously this is arbitrary because of fluctuations in *effective day-length* as a consequence of differences in meteorologic conditions. That such fluctuations may affect the rate of development is obvious from our unpublished data on k as a function of light intensity. These indicate that the maximal intensity is about four foot candles whereas the minimal intensity, if such exists, is somewhat less than one foot candle. There is the additional variable of the amount of cover about the bird early in the morning and late in the evening. Our measurements of light intensities in the Snake River Canyon suggest that the period between sunrise and sunset is about as satisfactory an approximation as we can select. A discussion of the approximate magnitude of error which could be attributable to this selection of values for p is included subsequently.

A Comparison of the Actual and Predicted Curves of Vernal Testicular Development

A comparison of the predicted and natural courses of vernal testicular development is effected in Figure 4 by plotting testicular weights of birds taken in the Snake River Canyon up to the time of migration during the springs of 1952, 1953, 1955, and 1956. These data indicate that the calculations based on laboratory-established rates predict the attainment of 100-mg. testicular weights about 10 days prior to the actual time of attainment in nature. This relatively close conformance is consistent with the hypothesis that the increasing vernal photoperiod is the primary timer in the annual development of the testicular cycle. However, it is of importance to examine the possible bases for this relatively small discrepancy between the calculated and actual curves. The possible bases are both statistical and natural.

(1) *The statistical factors.* A readily apparent possible cause of the differences between the calculated and actual curves in Figure 4 is the statistical nature of the initial testicular weights employed in the calculations, 0.7 mg. for first-year birds and 1.4 mg. for adults. These are mean values for birds taken in the field during the appropriate period. The logarithms are -0.17 ± 0.16 and $+0.15 \pm 0.19$, respectively. An inspection of Figure 4 indicates that a good conformance would have occurred had the selected initial weights been about one standard deviation below the estimated means. Also important is the possible error in weighing small testes since the balance and method used in weighing have a combined error of ± 0.1 mg.

Another possible factor is the statistical nature of the slope of the line relating k as a function of p. The curve in Figure 3 has been drawn by inspection with attention both to the means and their approximate 95% fiducial limits. It can be noted that a reasonable conformance between the predicted curve and the natural data would require the slope constant a in Figure 3 and equation (2) to be 0.007 instead of 0.009. Since this appears somewhat improbable, an error in a, although possibly contributory, cannot be regarded as the primary cause of the difference. It is also possible that the functional relationship between W and t with constant daily photoperiods may differ further from the relationship in equation (1) than our data suggest. An examination of models indicates that it is very unlikely that this could be the primary source of the difference. Obviously it could be contributory.

A further possible source of error is in the value of the minimal stimulatory day-length (q), or in our assumptions concerning the relationship of k to p and the value of p where k becomes insignificantly small. However, as noted above, since very small values of k are involved such errors affect the calculated curve only slightly.

There is also the possibility of error with respect to the effect of environmental temperature as indicated by c in equation (3). As noted previously the low-temperature group which was used in the estimation of c showed considerable variability with relatively wide fiducial limits for k. If the true k were close to the lower 95% fiducial limit, c would be about 0.019 and the 0° C. curves in Figure 4 would reach the 100-mg. level about 18 days later than the 20° curves, or about nine days later than shown in Figure 4. This would give a better, although not a complete, conformance. In other words, the statistical nature of c is such that it could contribute to the discrepancy although it is highly unlikely that it could be the sole basis for it.

(2) *The natural factors.* A possible source of the difference between the predicted curve and the natural data, as indicated above, lies in our selection of values for the effective day-length, p. The complexity of this problem has been discussed well by Bartholomew (1949). As a test of the magnitude of this source of error, we have assumed that p for each day is 30 minutes less than the period between sunrise and sunset. This alteration is almost sufficient to make the predicted curve coincide with the natural data. However, our observations on light intensities in the Snake River Canyon indicate that this assumption is unreasonable, for only rarely would p be as much as 30 minutes less than the period from sunrise to sunset; more frequently it would be somewhat greater than this period. Therefore, although our definition of p may be the source of some error, it is neither of sufficient magnitude nor in the right direction to account for the difference.

TABLE I

A comparison of body weights of male white-crowned sparrows in large outdoor aviaries at Pullman with those taken from the Snake River Canyon

Period	Snake River Canyon			In cages at Pullman		
	No.	Weight, grams	Standard Deviation	No.	Weight, grams	Standard Deviation
1–15 January	37	27.8*	±1.5	43	27.1*	±2.1
16–31 January	128	28.0	±1.6	51	27.8	±1.9
1–14 February	67	27.8**	±1.4	53	26.6**	±1.5
15–28 February	38	26.9	±1.2	45	27.5	±1.6
Prenuptial molt	55	27.8	±1.5	28	28.0	±2.8

* Significantly different, $P = ca.\ 0.04$.
** Significantly different, $P < 0.001$.

The estimates of k were affected with birds which had a nutritionally adequate food constantly available. The possibility exists that a nutritional difference could be involved in the differences between the calculated and actual courses of testicular development. That this possibility warrants consideration is evident from the demonstrated reduction of production of gonadotropic hormones by mammals in nutritionally inadequate states, as summarized by Ershoff (1952). Whether periods of nutritional insufficiency occur for white-crowned sparrows in the Snake River Canyon is obviously difficult to ascertain. However, a comparison can be made between the body weights of birds retained for a month or more under outdoor conditions in large aviaries at Pullman and those of birds taken from the natural population in the Snake River Canyon. The former received the same food *ad libitum* as did the experimental birds from which our laboratory-established rates were obtained. These data are summarized in Table I; it is obvious that they in no way support a hypothesis of poorer nutritional state among the wild birds. A further argument against an effective difference in nutritional state comes from a series of 29 males held in large outdoor aviaries at Pullman. The course of testicular development for this group shows no apparent difference from that of the natural population despite the constant availability of nutritionally adequate food. Although we cannot reject completely the possibility of an interaction between low temperature and poor nutritional state, certainly we find no evidence in our data to support it.

Discussion

It is patent that the greatest of caution must be applied in any inductive extrapolation of our experience with a single population of a single species. Nevertheless it may be of some value to consider briefly some possible bases for variations among the temporal patterns in the initiation of the testicular cycles of the temperate-zone passerine species in which day-length is the primary timer. In this discussion it is assumed that the basic relationships described here for *Z. l. gambelii* constitute, in a general way, a typical scheme.

It then becomes desirable to consider the general form of equation (2), relating day-length (p), the minimum effective day-length (q), and the rate constant (k):

$$k = a(p - q). \tag{7}$$

For arguments presented here, days (in winter) where $p < q$ must be treated as though $p = q$ and $k = 0$. This may be unrealistic, the true situation possibly being, as suggested above, that k may be a continuous positive function of p becoming insignificantly small as p becomes somewhat smaller than q. Our data are not inconsistent with this possibility. The data of Burger (1953) for *S. vulgaris* suggest this possibility. Although the effective difference between these two interpretations of the functional relationship between p and k is relatively trivial for *Z. l. gambelii* this may not necessarily be the case for other species. It is obvious that one way in which testicular maturation can be attained earlier in the year is with a lower value of q, or with a stronger curvilinear relationship between p and k in the lower tail of the curve. It is also obvious that when q is less than the shortest winter day, or, in the alternative interpretation of the relationship between p and k, when k has an appreciable value at the lowest winter values of p, the time of termination of the refractory period may become important with respect to the curve of testicular development. It seems possible that this could be applicable to certain non-migratory British species in which abortive fall and midwinter sexual activity has been noted (Marshall, 1949, 1952). An essential part of such a hypothesis would be a more marked relationship between k and the temperature coefficient (c).

The literature actually contains very little useful data on the minimum effective day-length (q). For *S. vulgaris* it appears to be less than 8.5 hours (Burger, 1949). A recalculation of the data of Bartholomew (1949, Fig. 15) suggests that q is about nine hours for *P. domesticus*. However, because, in another experiment his data (p. 444) contain two cases of at least some histologic development at 8-hour photoperiods, it is possible that the alternative interpretation of the relationship between p and k may hold. In considering the matter of the minimum effective day-length (q), it is necessary to bear in mind the possibility of the existence of an internal timer as Miller (1955) has suggested for *Zonotrichia coronata* and

Zonotrichia leucophrys nuttalli. The data of Benoit *et al.* (1955) suggest a similar possibility in ducks.

Another way in which the time of maturation of the testes may be affected is by the rate of testicular growth (k) once stimulatory day-lengths occur. As indicated in equation (7), a greater slope constant a will result in a more rapid development. From the data of Bartholomew (1949) for *P. domesticus* it has been possible to calculate a curve relating k to p. In this calculation it has been assumed that the rate of testicular growth under the influence of daily photoperiods of constant length is in accordance with the logarithmic curve expressed by equation (1). Values of k for several different photoperiods have been similarly calculated from data obtained for this species in our laboratory and from Vaugien (1952). These values are consistent with the curve derived from Bartholomew's data. The curve is similar to that of *Z. l. gambelii* (Fig. 3); however, the linear part is steeper, a being 0.013 compared to 0.009 and 0.008 for first-year and adult *Z. l. gambelii.* It appears, then, that the greater value of a for *P. domesticus* correlates well with the earlier testicular development of this species in nature. It should be observed that direct comparisons of a for different species are meaningful only when the differences between the logarithms of the resting and developed testes are very similar. This is essentially true for *Z. l. gambelii* and *P. domesticus.*

It should also be noted that a greater adjustment to the conditions of the developing season could be obtained with a greater sensitivity to environmental temperature as indicated in equation (3). As c becomes greater the time of maturation would fluctuate more as a function of environmental temperature. This would be particularly valuable to an early breeding, non-migratory species, but also of some value to species whose migratory route is confined to a relatively restricted range of temperate-zone latitude. As noted above, it would be very interesting to investigate the British species discussed by Marshall (1949, 1952) with respect to temperature sensitivity, the termination of the refractory period, and the functional relationship between p and k.

It is obvious that variations in the values of a, c, q (or the nature of the lower tail of the curve relating k to p), and the terminal dates of the refractory periods could produce a wide range of times at which testes mature. However, it must be re-emphasized that these arguments are derived almost exclusively by extrapolation from experiments on a single population of *Z. l. gambelii.* It would be of great interest to examine additional species similarly.

SUMMARY

1. The rate of testicular development in *Zonotrichia leucophrys gambelii* has been examined quantitatively as functions of day-length, light intensity, and environmental temperature.

2. From these laboratory-established relations a predicted curve for testicular growth under natural conditions was calculated and compared with data obtained from a natural population. The predicted curve indicates the attainment of 100-mg. combined testicular weight about ten days earlier than its occurrence in the natural population. Although this relatively small discrepancy may be reasonably explained on a statistical basis, it is not possible to rule out minor effects by environmental variables other than daily photoperiod and temperature.

3. The calculations, and the relatively close agreement with the observations of the natural population, emphasize quantitatively the overwhelming importance of the daily photoperiod as the primary timer in the testicular cycle for this population of *Zonotrichia leucophrys gambelii*. Other environmental factors, as they operate in the Snake River Canyon, appear to be responsible for fluctuations with a combined maximum possible magnitude of the order of ten days to two weeks.

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A BEHAVIORAL MECHANISM FOR OSMOTIC REGULATION IN A SEMI-TERRESTRIAL CRAB

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It is generally known that an animal can regulate osmotically by possessing a relatively impermeable integument and by utilizing specialized organs which by active metabolic processes control the flux of salts and water between animal and its external medium. However, there is evidence that animals are capable of assisting the osmo-regulatory mechanism by their behavior.

Barnes (1935, 1938, 1940) demonstrated that the isopod, *Ligia baudiniana*, prefers distilled water to normal sea water when the choice is offered in the form of moistened filter paper. Gross (1955) revealed that the land crab, *Birgus latro*, can control the osmotic pressure of its body fluids by selection of appropriate sea water concentrations. Krijgsman and Krijgsman (1954) have produced evidence of an osmo-receptor in the spiny lobster *Jasus* which apparently serves to guide the animal with respect to salinities.

Pachygrapsus crassipes is a crab of semi-terrestrial habits, most often found in exposed, rocky shore situations, but also occasionally found in protected bays in muddy burrows. Typically, it does not inhabit waters deviating much in salinity from normal sea water, but it can regulate osmotically in both dilute and concentrated sea water (Jones, 1941; Gross, 1955; Prosser *et al.*, 1955). Gross (1955) raised the question as to the adaptive significance of strong osmo-regulatory powers in this crab which is extremely agile and easily capable of reaching normal sea water should it by chance venture from the sea into an osmotic stress such as would be afforded by an isolated, evaporated tide pool, or one diluted by rain water.

In Southern California, situations affording such osmotic stresses are rare. However, there undoubtedly are regions in this crab's range where osmotic stresses are readily enough available so that selection would favor the development of an osmo-regulatory mechanism. On the other hand, should *Pachygrapsus* be capable of detecting salinities deviating from normal sea water and should it shun abnormal salinities, then the adaptive significance of osmotic regulation in this species could remain in doubt.

The present investigation will show that *Pachygrapsus* does show preference for sea water of normal salinity.

Materials and Methods

Crabs of the species *Pachygrapsus crassipes*, collected at Laguna and Newport, California, were placed singly in a box in which there were four containers, each containing sea water of a different salinity. The containers were placed in each corner of the box and sunk so that their rims were flush with the platform which constituted a second floor of the box. In each container was a treadle which could be depressed by about 15 grams. The surface of the treadle was barely below the water level in the container.

As a crab entered a given medium, it was necessary for it to depress the treadle. This caused the deflection of a signal magnet which was recorded on a kymograph drum, rotating at a known rate. Thus a crab placed in the box had the choice of remaining on the platform in the air or entering any of the containers in each of the four corners of the box. The kymograph record then would indicate the movements of the crab while it remained in the box, what container it entered, when it entered and how long it remained.

The selection box was placed in a darkened, quiet, temperature-controlled room (20° C.). As a control against selection for any given corner, rather than salinity, the respective sea water concentrations were rotated in position with each successive test specimen. In most cases only three containers were filled with water; the fourth remained empty to check on the preference for corners. Each crab recorded, remained in the box for at least 40 hours.

Sodium and potassium concentrations of the blood were determined in a few cases by means of a Beckman flame photometer.

RESULTS

Table I summarizes the results of the following experiments:

1. Crabs freshly removed from normal sea water were given a choice of 50, 100, or 150% sea water (seven crabs).

TABLE I

Salinity preference in Pachygrapsus crassipes

Treatment	Mean time spent in selected salinities										
	50% sea water		75% sea water		100% sea water		125% sea water		150% sea water		Time spent out of water (%)
	% time	time (hr.) / No. visits	% time	time (hr.) / No. visits	% time	time (hr.) / No. visits	% time	time (hr.) / No. visits	% time	time (hr.) / No. visits	
Normal (7 animals)	6.2	$\frac{3}{32}$			29.7	$\frac{16}{55}$			3.7	$\frac{2}{29}$	59.7
Normal (5 animals)			6.1	$\frac{3}{12}$	39.6	$\frac{17}{49}$	6.9	$\frac{3}{24}$			47.5
Acclimated to 50% sea water (5 animals)	7.7	$\frac{4}{42}$			29.8	$\frac{15}{37}$			7.3	$\frac{4}{33}$	55.4
Acclimated to 150% sea water (5 animals)	13.5	$\frac{7}{32}$			16.1	$\frac{9}{22}$			10.1	$\frac{6}{21}$	60.3
Desiccated (6 animals)	11.4	$\frac{8}{51}$			47.6	$\frac{29}{54}$			3.6	$\frac{2}{32}$	37.5

Legend: Normal = animals freshly removed from normal sea water;
% time = % total time spent in chamber.

2. Crabs freshly removed from normal sea water were given a choice of 75, 100 or 125% sea water (five crabs).

3. Crabs acclimatized to 50% sea water 2–7 days were given a choice of 50, 100 or 150% sea water (five crabs).

4. Crabs acclimatized to 150% sea water 1–3 days were given a choice of 50, 100 or 150% sea water (five crabs).

5. Crabs desiccated 2–4 days were given a choice of 50, 100 or 150% sea water (six crabs).

Of the above 28 specimens studied in the selection chamber, 25 spent more time in 100% sea water than any salinity offered, thus indicating that *Pachygrapsus* does show a decided preference for normal sea water. Two of the above exceptions were among the group which had been acclimated to 150% sea water. The third was one of those which had been desiccated. Although Table I shows that animals first acclimatized to 150% sea water average more time in 100% sea water than any other salinity, the histories of the individuals do not show such a preference.

All six of the crabs which were desiccated before being placed in the box remained in water longer than in the air, which might be expected. However, of the remaining 22 test crabs, 17 spent more time out of the water than in all salinities offered, thus suggesting the degree of the aerial habit in this marine form.

Considering individual histories, 14 crabs spent more time in the hypotonic medium than in the hypertonic medium. Six spent more time in the concentrated media and eight showed no significant preference between the two types of stresses. It is interesting, however, that of the 14 preferring dilute to concentrated media, four had been previously acclimated to 150% sea water, only one of which preferred dilute to normal sea water. Three of the above 14 had been desiccated previously, one of which showed a preference for dilute sea water over normal sea water. With the sample at hand, nothing can be said concerning the preference of dilute and concentrated sea water or vice versa.

In only one of the 24 cases where one of the four containers was not filled with water did a crab spend more time in the empty container than it did on the platform between the containers, *i.e.*, out of the water. Thus, there does not seem to be any particular preference for corners, at least in an aerial situation.

Of 25 crabs showing preference for 100% sea water, judging by the time they spent in this salinity, 14 made more visits to normal sea water than any other offered aqueous medium. On the other hand, 11 made more or an equal number of visits to other than 100% sea water. Thus, with the sample at hand, nothing can be resolved precisely as to the learning process involved.

Table II shows the blood sodium and potassium concentrations of some of the crabs which had spent at least 40 hours in the selectivity chamber. Here it can be seen that the blood sodium of one of the five crabs which was first acclimated to 150% sea water (number 4) was definitely above the normal concentration. It should be pointed out, however, that this particular specimen was somewhat weakened by its previous treatment in 150% sea water and this was the only crab that spent more time in concentrated sea water than in any of the other salinities. Thus, four out of five specimens in the group attained close to normal blood concentrations after a period in the selectivity chamber. The blood sodium concentration of two out of the three of the desiccated group sampled was below the normal concentration range. This is particularly interesting in the case of specimen num-

TABLE II

Blood sodium and potassium concentrations in Pachygrapsus following period in selection chamber

Specimen No.	Treatment	Blood concentration after treatment*	
		Na (meq/l)	K (meq/l)
1	Normal	484	6.35
2	Acclimatized to 50% sea water	498	—
3		481	8.08
4	Acclimatized to 150% sea water	544	7.72
5		477	6.62
6		477	7.66
7		495	8.81
8		498	8.06
9	Desiccated	417	7.35
10		479	8.50
11		414	10.4
95% fiducial limits**		478–489	7.19–7.67

* Concentration choices available for all specimens were 50, 100, and 150%.
** Calculated from observations on 36 crabs freshly removed from the sea.

ber 9 which spent 51% of its time in 100% sea water while it was in the chamber. Specimen number 11, however, spent more time in 50% sea water than in any other offered salinity. The remaining cases shown in Table II are within or close to the normal blood concentrations with respect to sodium.

Considering the intrinsic error in determining potassium concentrations by the methods used in this investigation (about 10% of the concentration of normal blood), only the blood potassium of specimen 11 (Table II) seems to vary from normal. This, however, would have little effect on the osmotic pressure of the blood.

The activity in the selection chamber of the above crabs also was considered with respect to the time of day and phase of the tide, but no periodicity could be resolved.

DISCUSSION

Using duration of immersion as a criterion, *Pachygrapsus* prefers normal sea water to hypotonic or hypertonic media varying at least 25% from 100% sea water. It thus seems that in a natural situation, this crab probably would not remain long in an osmotic stress, but soon would return to the salinities of normal sea water. *Pachygrapsus* possesses a relatively impermeable integument (Gross, 1957) and this means that there would be a strong passive resistance to salt-water exchanges, should the crab enter an osmotic stress. The question is posed then as to the need for osmotic regulation in an animal which is rarely exposed to such stresses and which would attempt to escape, and could escape to the comfort of the nearby sea should the occasion arise.

The strong preference for normal sea water, it would seem, would be a powerful factor tending to restrict this species to the intertidal and subtidal zones of the sea. For example, a period of desiccation, such as might be encountered by remaining out of the water, would stimulate the return to the water, but as suggested above, not just to any water, but to normal sea water. Again, in an estuarine situation, where a sharp salinity gradient would be available, an animal responding thus to osmotic stresses would tend to avoid brackish water, even though it possessed the ability to regulate strongly in such stresses. Even in a case where a population were temporarily trapped in a dilute situation for an extended period, this period of acclimation, as shown above (Table I), does not seem to diminish the preference for normal sea water.

Of course, the distaste for brackish water possibly could be overcome by other factors, *e.g.,* food source and retreat from predators.

Acclimation to 150% sea water apparently breaks down the preference for normal sea water (Table I), although the fact that four out of five of the specimens thus treated were able to achieve normal blood concentrations suggests effective salinity selection of a sort. This preference breakdown perhaps is correlated with the ability of *Pachygrapsus* to regulate more strongly in dilute than in concentrated media (Jones, 1941; Gross, 1955; Prosser *et al.,* 1955). In such case, a period of acclimation would necessitate a greater alteration on the physiological condition of the organism and consequently a greater effect on its behavior. Then, too, a precise "knowledge" of external salinities possibly is necessary for adequate regulation. Should a period of immersion in 150% sea water reduce the accuracy of the appropriate receptors, whatever they may be, and efficiency of such receptors were necessary for regulation, then it would follow that *Pachygrapsus* would show relatively weak regulation in the concentrated sea water.

On the other hand, as shown above, acclimation to 50% sea water does not appreciably affect the preference for normal sea water. This may be simply because *Pachygrapsus* can regulate strongly in dilute media and the consequent physiological alteration in the crab would be held at a minimum. Or the hypotonic media may not reduce the efficiency of the osmo-receptors and the organism, being "aware" of the external salinity, could regulate accordingly.

It seems, then, that should a population of *Pachygrapsus* be confined to a hypertonic medium for an extended period, by losing its preference for normal sea water, it might remain in such concentrated environments, assuming all other biological requirements were satisfied. However, it is difficult to imagine natural hypertonic situations capable of isolating *Pachygrapsus* from the sea.

Table II demonstrates the general tendency for *Pachygrapsus* to achieve normal blood sodium concentrations after a period in the selectivity chamber. This seems to be true even when blood concentrations were forced away from normal by acclimation to 50% and 150% sea water. Two exceptions, however, were crabs previously desiccated. The final blood concentrations in these cases were lower than normal, thus suggesting over-compensation.

It has been demonstrated numerous times that organisms immersed in stress media metabolize more rapidly than when they are in their normal media (Schlieper, 1929; Schwabe, 1933; Flemister and Flemister, 1951). This increased metabolism has been interpreted often as added osmotic work. However, doubts have been thrown on this interpretation (Krogh, 1939; Wikgren, 1953; Potts, 1954).

Gross (1957) reports that crabs immersed in stress media apparently attempt to escape and thus become more active. He suggested, then, that increased oxygen consumption in increased osmotic stresses was merely the reflection of the attempt to escape an uncomfortable medium. The preference for normal sea water established quantitatively by the present investigation corroborates this suggestion.

With the exception of those crabs first desiccated, the experimental animals spent about half their time out of water. This suggests a high degree of adaptation to the aerial habit, but not so much compared with the land crab *Birgus* which under experimental conditions spends only about one hour per day visiting water (Gross, 1955).

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SUMMARY

1. The shore crab *Pachygrapsus crassipes* prefers 100% sea water to 50, 75, 125 and 150% sea water.

2. This preference could not be altered by first desiccating the animal or by acclimating the animal for several days in 50% sea water. The preference could be altered somewhat by acclimating to 150% sea water.

3. The preference for normal sea water by *Pachygrapsus* suggests a mechanism which tends to restrict this crab to the intertidal and subtidal zones of the sea.

4. *Pachygrapsus* under the experimental conditions of the present investigation spends about 12 hours per day visiting water. This compares to one hour per day for the land crab *Birgus*.

5. *Pachygrapsus* tends to maintain normal sodium and potassium blood concentrations when given free choice of salinities, including 100% sea water. Normal concentrations are generally achieved under the same conditions even when the blood has been forced away from normal by acclimation in 50 or 150% sea water. However, animals previously desiccated may over-compensate when offered a choice of media varying in salinity and consequently achieve blood sodium concentrations below the normal range.

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FREEZING AND DRYING IN INTERTIDAL ALGAE[1]

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The intertidal region is an environment characterized by widely fluctuating conditions. It will be shown in this paper that in high latitudes the algae in this zone are exposed to extensive freezing and drying. These two aspects of immersion are considered together, since they have the common feature of cellular dehydration. In particular, their separate effects on the metabolism of algae have been investigated.

First will be discussed the effect of low temperatures in freezing a large amount of water in certain algae. Next, the natural dehydration that is caused by evaporation in several of the same species will be described. Finally, measurements will be reported which show a greatly depressed respiration in both the frozen and dried states. Some observations on the winter survival of *Fucus* in the Arctic are also included.

I. FREEZING

In the Woods Hole region in winter there are a number of macroscopic brown, green, and red algae exposed to freezing temperatures by the tide. They frequently feel brittle to a degree which suggests ice in them. Some of them have been observed imbedded in the ice at temperatures as low as − 20° C. Conditions are even more rigorous in the Arctic where *Fucus* is a prominent intertidal alga. It may spend six months or more frozen in the ice at temperatures which go below − 40° C. Since these plants contain 70 to 80 per cent water, it seemed pertinent to determine how much of this water, if any, is frozen at these extreme temperatures. Bieble (1939) reported that several intertidal algae would survive being frozen, but he made no quantitative determinations of the ice.

1. *Method*

Water gives off heat and also expands when it changes to ice. The expansion has been measured directly in a dilatometer by Moran (1935), Gortner (1937), and others to determine the amount of ice. Scholander *et al.* (1955) devised a flotation method to measure specific gravity and found as much as 90 per cent frozen water in the lichen *Cetraria richardsonii*. Volumetric methods were unsuitable here because of the difficulty of the dissolved gases that come out of solution on freezing. This would cause a density change which could not be separated from the same effect due to ice formation. Calorimetric determinations of ice, based on the conveniently large heat of fusion of water, have been used by Greathouse (1935), Ditman *et al.* (1942), and others. Scholander *et al.* (1953) have recently reviewed these two methods.

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There has been no previous quantitative work on ice in algae. This author has described a simplified calorimetry for small animals (Kanwisher, 1955) which has been used here on several of the large intertidal algae. The calorimeter vessel is an ordinary Thermos bottle. The temperature of the water inside is read to 0.01° C. by a mercury thermometer through the stopper. The sensitivity is varied by changing the amount of water in the Thermos. Weighed pieces of ice are used for calibration.

A piece of the alga to be frozen, usually a few grams, is sponged free of excess water with filter paper, weighed, and placed in a small vial in a cold chamber set to the desired temperature. At least four hours is allowed for phase equilibrium between the water and ice in the alga. A check weighing at this point usually showed less than 1 per cent loss of water by evaporation.

The Thermos is thermally equilibrated with the desired amount of water and the temperature noted. Then the vial is removed from the cold chamber and alga immediately shaken into the open Thermos. The vial prevents transfer of heat from the hands to the frozen alga. The stopper is replaced quickly and the Thermos is shaken. During the next few minutes the lowest temperature is noted. The measurement is completed by weighing the alga after drying it for two hours in an oven set at 100° C. The amount of water and dry substance in the initially frozen material is then computed.

If no ice is formed the number of calories supplied to the alga is proportional only to its weight and the temperature interval through which it is warmed. In the absence of any change of state the specific heat is nearly constant with temperature. If ice is present the calorimeter must supply 80 additional calories to melt each gram.

The calories supplied to the alga by the calorimeter are equal to the temperature drop observed times the heat capacity which has been determined by calibration with ice. Part of these calories go to warm the dry substance and the water from the cold box temperature T_1 up to the final calorimeter temperature T_2. This is equal to

$$(T_2 - T_1) \times (0.3 \times \text{dry wt.} + \text{wt. of water}),$$

where 0.3 has been separately determined to be the specific heat of the dry substance. That of water is 1.0. The remainder of the calories melt any ice that is present. This is converted to grams of ice. Since the water in the starting sample is known, the fraction of it frozen at the temperature T_1 has been determined. A small correction is necessary because the specific heat of ice is only half that of water (Ditman *et al.*, 1942).

2. *Results*

Figure 1 is typical of the data thus obtained. It is a plot of the per cent of water that is converted to ice at various freezing temperatures in *Fucus vesiculosus*. Similar curves were obtained for *Ascophyllum nodosum, Chondrus crispus*, and *Ulva lactuca*. The following table shows the percentage of water as ice at −15° C., the lowest temperature used.

It is evident that a large fraction of algal water is readily frozen at temperatures which frequently occur in nature. The large surface area of *Ulva* would tend

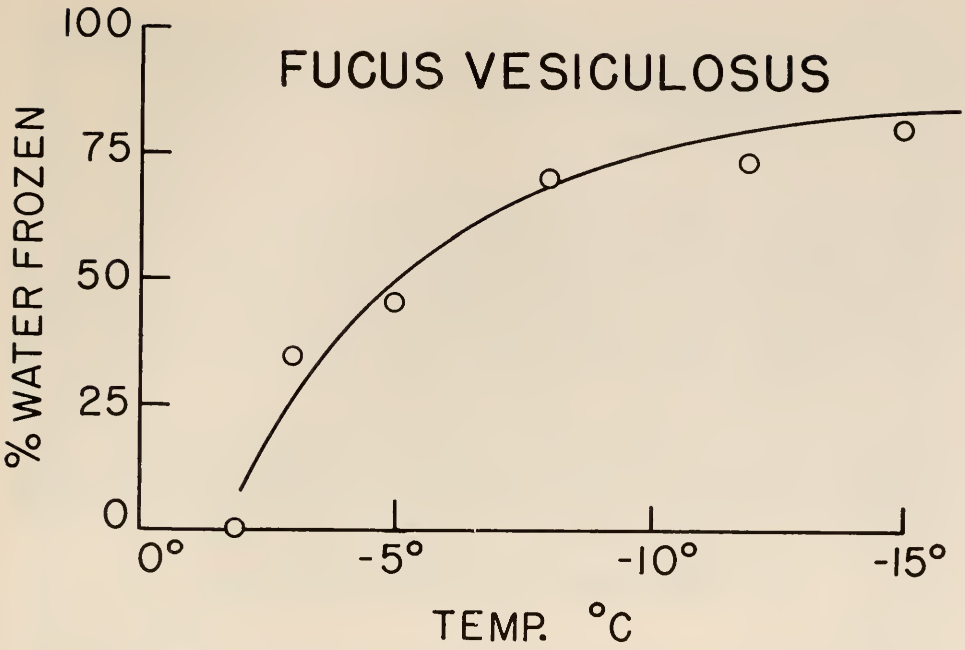

Figure 1. Freezing curve of *Fucus*.

to absorb heat during the transfer to the calorimeter. This may account for its having the least ice in Table I.

II. Water Content in Algae Naturally Dried

Exposed upper intertidal algae become very obviously dried on a windy day when the relative humidity is low. Isaac (1933) measured a 68 per cent loss in weight in *Pelvetia canaliculata* during normal exposure on the short. He did not determine the dry weight. Feldmann (1937) noted that *Bangia fuscopurpurea* can remain out of the water for periods as long as 15 days and still survive. Zaneveld (1937) found only a 30 per cent weight loss in drying conditions. A series of measurements have been made here to determine how much water is normally lost under such conditions.

When the algae looked very dry at low tide on a windy day, samples were taken and weighed immediately. They were then immersed in sea water for several hours and weighed again. Some of them were used to demonstrate photosynthesis by a method described later. Finally, oven drying and weighing gave the necessary

Table I

Species	Per cent ice at −15° C.
Fucus vesiculosus	82
Ascophyllum nodosum	76
Chondrus crispus	74
Ulva lactuca	69

TABLE II

Species	Per cent of water lost
Fucus vesiculosus	91
Chondrus crispus	63
Ulva lactuca	77
Enteromorpha linza	84

data to compute the water present when immersed and also in the dried state. Table II gives the maximum dehydration values found.

To measure the rate of loss and reabsorption of water, pieces of *Fucus* were exposed to room air of 22° C. and 40 per cent relative humidity. Such values are not uncommon in nature. They were weighed at intervals and then replaced in sea water and their weight again followed. From Figure 2 it is clear that severe drying can take place during the length of time of tidal exposure. The rate was increased several fold when a breeze was simulated with an electric fan. Less than an hour after re-immersion most of the lost water has been regained. Thin forms such as *Ulva* probably dry even more rapidly. Where the algae grow in many overlapping layers probably only the uppermost are dried very much. The interest here, however, is only in the maximum drying that can be tolerated.

III. RESPIRATION IN FROZEN AND DRIED ALGAE

1. *Method*

Respiration is awkward to measure at freezing temperatures with conventional methods such as the familiar manometric technique. The volume change resulting from water turning to ice cannot readily be separated from that due to oxygen consumption. The Winkler method of following the disappearance of dissolved

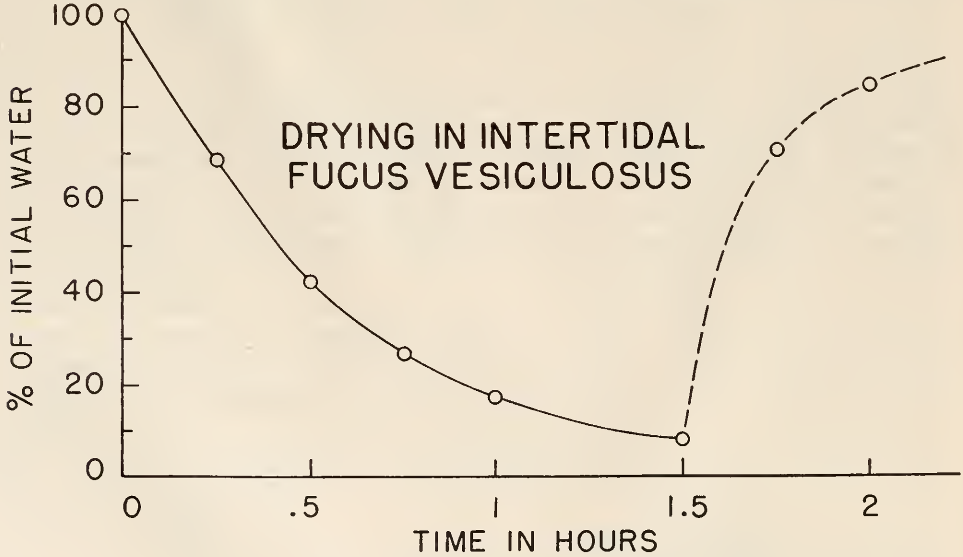

FIGURE 2. Drying curve of *Fucus*.

oxygen cannot, of course, be applied to frozen or dried material. Oxygen consumption in the region of freezing is so sharply temperature-dependent that any method which requires part of the time at higher temperatures is open to gross errors. Scholander *et al.* (1953), in reviewing the literature on respiration in frozen material, concluded that many of the techniques were inadequate for the problem at hand. They used a method of gas analysis which has been applied here with slight modification.

The material is enclosed in a syringe with a known amount of air and kept in the dark. Samples of gas are withdrawn at intervals and analyzed for oxygen. Respiration is computed from the rate of oxygen decrease in the gas phase. Since blanks run in the same way give negligible values, the utilization of oxygen can only be attributed to the frozen or dried algae. The method is specific for oxygen and does not rely on volume decrements which are assumed to result from oxygen being used.

For the low temperature values the algae were placed in darkened hypodermic syringes in a cold bath set at the desired temperature. At least six hours was allowed for the ice-water equilibrium to be reached. The syringes were flushed with chilled outside air and sealed. The tips of the syringes extended above the surface of the coolant. Samples of the gas could be removed without taking the syringes from the bath. The plunger was free to move up as a sample was withdrawn. By thus avoiding any differential pressure when sampling, the danger of leaks is reduced.

The oxygen was measured with the ½-cc. gas analyzer of Scholander (1947) to 0.02 per cent accuracy. Duplicate analyses were made. When a respiratory period was started by flushing in outside air, the initial concentration was assumed to be 20.94 per cent. Repeated measurements varied between 20.93 and 20.96. Because of the possibility of rate of oxygen consumption depending on tension, oxygen was never depleted below 18 per cent. Two readings were usually taken. The time between these varied from 1 to 200 hours. The slope determined by these points was used to compute the rate of oxygen consumption. The figures are in units of mm.^{3} 0_2 per gram of dry weight of alga per hour.

For the dried algae a fresh sample was weighed fully moist and dried by exposing it to air. It was then weighed again and placed in a syringe. The air in the syringe becomes saturated and no additional water is lost. The readings were made by the same method as for the frozen material. The temperature was kept at 15° C.

Fully moist respiration measurements above 0° were made with volumetric respirometers (Scholander *et al.*, 1952). At 0° these checked with the gas analysis method.

2. *Results*

Respiration in *Fucus* above and below 0° is plotted in Figure 3. Similar curves were found for *Chondrus* and *Ulva*. The respiration drops sharply below 0°. In in the interval from 0 to − 10 the apparent Q_{10} is 17. For the same interval above 0° it is 2. The other species had a Q_{10} of 15 and 23 below 0°, respectively, and close to 2 above. At − 15° it was necesary to wait 7 to 8 days for the oxygen in the syringe to decrease by a large enough amount to insure an accurate determination.

When the algae were dried, the oxygen consumption again decreased. Figure 4 shows the respiration of *Fucus* related to the degree of dehydration. When 80 per cent of the normal water was lost, the metabolism was down to one-sixth of its normal value. If a sample was allowed to regain water by soaking, the metabolism increased. The solid points were taken consecutively on the same piece of material and showed the reversible nature of the phenomenon. *Chondrus* and *Ulva* showed the same decreased respiration when dried and also recovered the higher rate when re-immersed.

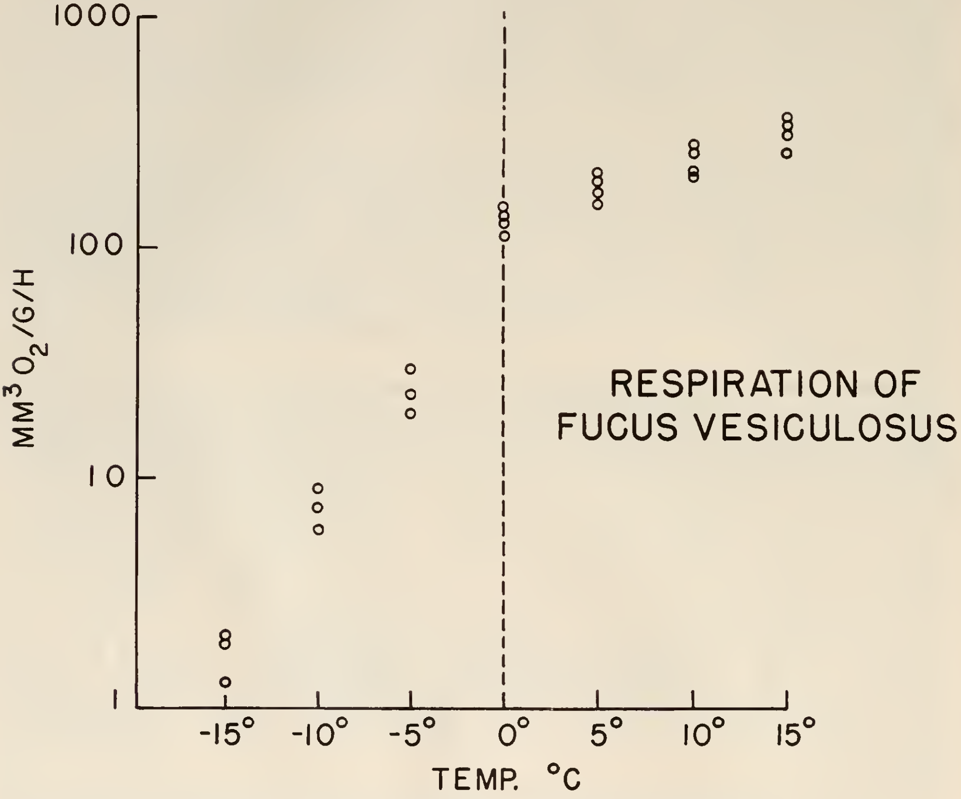

FIGURE 3. Oxygen consumption vs. temperature of *Fucus*.

Loss of water in freezing or drying must increase the salinity of the remaining fluids. Although part of this fluid is in the inter-cellular space, it is in equilibrium with the interior of the cells. Thus loss of any algal water will raise the salinity inside the cells. To determine whether salinity had a specific effect on metabolic rate, higher salinities were made by draining the brine from frozen sea water. Pieces of *Fucus, Chondrus,* and *Ulva* were immersed in these for 12 hours. The tissue chloride concentration was measured by acid digestion and titration. It was always proportional to that of the external medium. These species show no evidence of regulating chloride. Respiration rates were measured at the various salinities. In all cases oxygen consumption decreased less than 30 per cent when the salinity was increased by three times. This concentration is produced at − 8° C. by freezing out of water. At such a temperature the respiration is decreased 10- to 15-fold below that at 0°. Salinity is clearly not the primary respiratory depressant when water is lost from the cells by either freezing or drying.

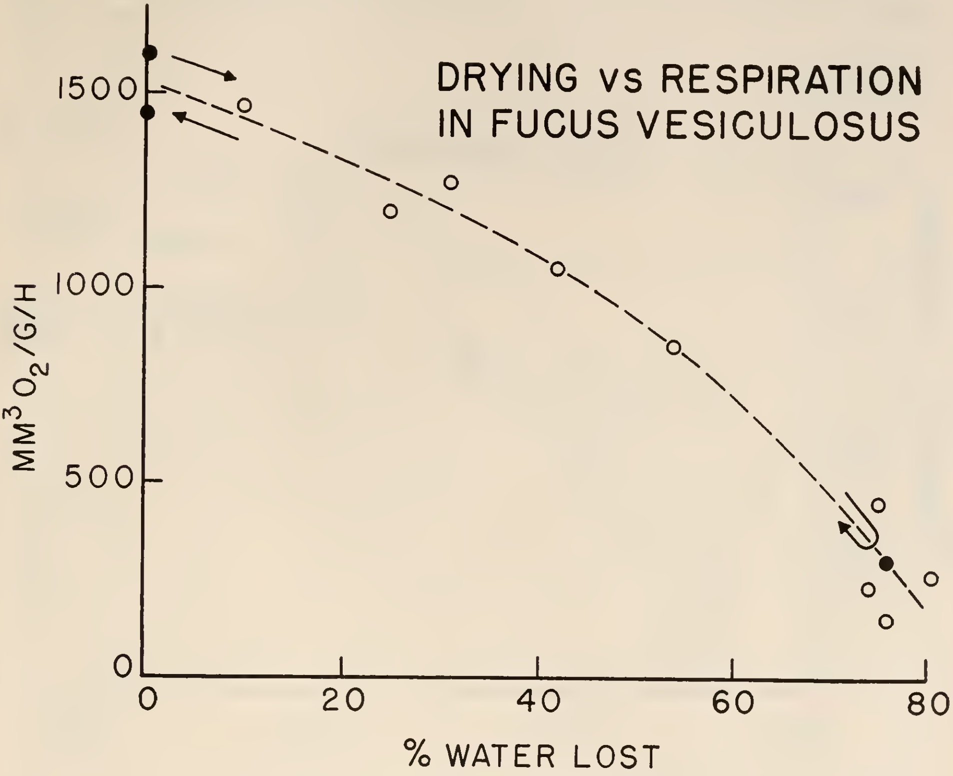

FIGURE 4. Oxygen consumption at various degrees of dehydration.

IV. DISCUSSION

Previous attempts have been made to determine respiratory gas exchange in frozen plants. Scholander *et al.* (1953) measured a precipitous drop in oxygen consumption of several Arctic phanerograms and lichens below 0° C. Ice determinations by a floatation method showed that at − 20° C., more than 90 per cent of the water in some lichens was frozen. They thought it likely the drop in metabolic rate was due to cellular dehydration. They have reviewed the literature and point out that the techniques used previously to measure low temperature respiration were inadequate for the low rates that occur. At freezing temperatures they found a Q_{10} of 20 to 50, while above 0° the same material showed the usual two- to four-times change in oxygen consumption over a ten-degree interval.

Smyth (1934) found a linear relationship between water content and respiration in lichens. The respiration of air-dried *Acacia* seeds was measured by White (1909) to be only 1/10,000 that of moistened seeds. In dry *Ricinus* seeds he could detect no oxygen consumption. Spores of single-celled forms are dehydrated and are known to show very low oxygen consumption. Respiration in dried algae does not seem to have been previously studied.

The intertidal algae used in these experiments are normally exposed to both freezing and drying. It has been shown here that their respiration under either of these conditions is sharply reduced. In the region of freezing temperatures their Q_{10} is about two times what would be expected from dehydration alone. In going from 0° to − 10°, 75 to 80 per cent of the water is frozen. This water loss alone should cause a decrease of six to ten times in oxygen consumption. When the straight temperature effect of a Q_{10} of two is also included, the total apparent Q_{10} should be in the range of 12 to 20. This is in reasonable agreement with those measured directly in the frozen algae.

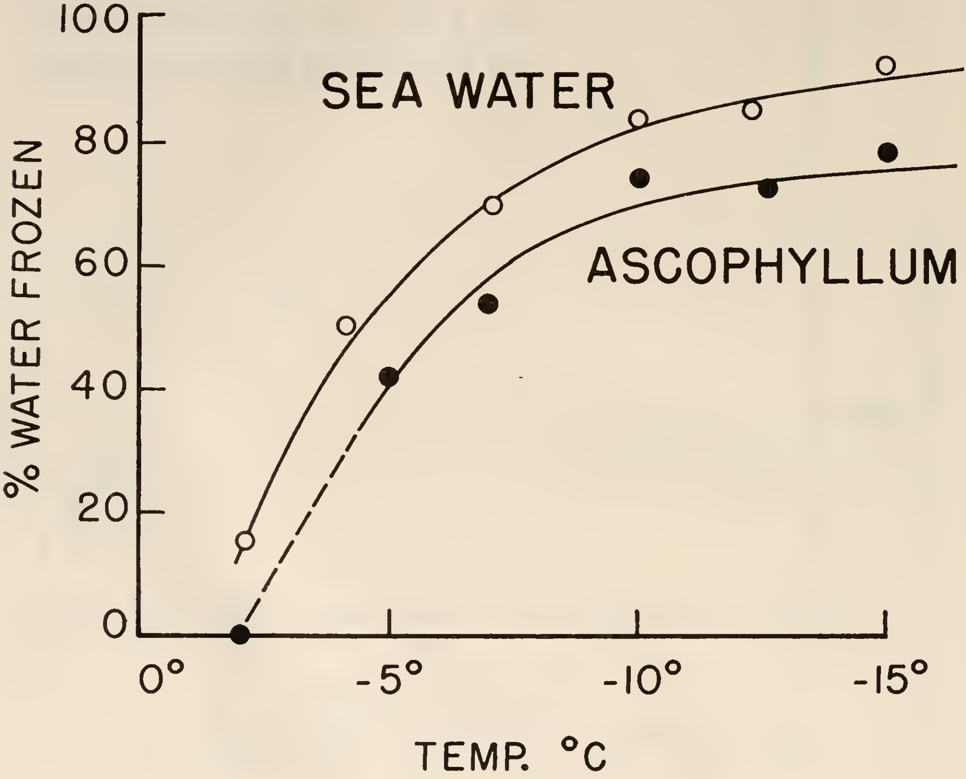

FIGURE 5. Curves showing the freezing of a greater fraction of sea water as compared to algal water.

At progressively lower temperatures calorimetric ice determinations become less accurate. More heat must be supplied to warm the material over the increased temperature range. The calories representing the melting of ice become a smaller fraction of the total measurement and are thus subject to a larger percentage error. It appears from Figure 1, however, that 15 to 20 per cent of the water is resistant to freezing. The same technique of calorimetry has been applied to a vial of sea water to find the amount of ice at various temperatures. It is compared with *Ascophyllum nodosum* in Figure 5. A larger fraction of the water in sea water freezes at all temperatures than in the algae.

It appears that part of the water in algal cells is unavailable for freezing. Many authors have presented evidence for unfreezable water in gels and also in plant and animal material (Moran, 1926; Greathouse, 1935; review of the literature by Scholander *et al.*, 1953). Moran was unable to freeze all of the water in a gelatin, even at − 40° C. The water molecule has a large dipole moment and may well be subject to forces less powerful than conventional bonding but still strong enough to prevent its being frozen. However, Grollman (1931) has rejected the idea of bound water in colloidal systems.

White (1909) found evidence for binding of part of the water in plants at ordinary temperatures. In *Acacia* seeds three per cent remained even after drying over calcium chloride.

Roualt's law expresses a linear relationship between the concentration of a solution and its freezing point depression. If one can assume that the ratio of dry matter to water is equivalent to a concentration, this quantity should increase linearly below freezing. Scholander *et al.* (1953) found this to be so in a *Chironomus* larva. Sea water is nearly linear as would be expected of a solution of crystalloids. The ratio in *Ascophyllum* tends towards a constant value at low temperatures. This could happen if part of the water were bound in such a way that it would not freeze. It would also result from any of the dissolved substances coming out of solution. The concentrations increase as water is frozen out while at the same time the solubility must decrease with temperature. In a frozen alga at − 15° C., the solubility of sodium chloride has been exceeded. The cells can either maintain a supersaturation or must be able to actually cope with internal salt crystals.

Siminovitch and Briggs (1949) measured an increased mobility of water in the frost-hardy cells of the black locust, *Robinia*. They thought this was necessary to allow a more rapid exit of water from the cells when intercellular freezing occurs. Such is likely the case with the algae used here when they undergo rapid freezing and drying. It is generally believed that internal freezing is lethal to cells, probably by the physical disruption of ice crystals in the protoplasm. Direct observation will be necessary, however, to determine the locus of this ice.

The lowered respiration observed in the frozen and dried algae may be of value to them in surviving these periods of stress. There can be little growth at such times since the usual supply of nutrients from the sea water is not available. When the alga is frozen, the light available for photosynthesis is usually limited, such as during the Arctic winter. The slowing-down observed here represents a less serious drain on the food stores. The ability of these algae to survive in the intertidal zone may, however, be merely a case of their not being injured by the freezing and drying that are inevitable in such a location.

Respiration has been called the flame of life. In algae at low temperatures it burns very low but is never entirely out.

V. OBSERVATIONS OF ARCTIC FUCUS

The author was a member of an expedition to Hebron in northern Labrador, sponsored by the Arctic Institute of North America in 1954. On arrival early in July, *Fucus* was abundant in the intertidal zone along much of the coast. Large reproducing plants were common although the ice had been gone only a month. It seemed certain they had not grown this much in the brief period of open water. Yet during the winter the ice is several feet thick and is solid to the bottom along the shore. One was led to believe the *Fucus* was frozen solid in the ice during the entire winter.

During this summer visit, pieces of *Fucus* were cooled to − 13° C. in a vial immersed in a salt and ice mixture. Calorimetry showed that ¾ of the water was frozen at this temperature. About two grams wet weight of recently frozen *Fucus* were put in a 20-cc. syringe with sea water. The syringe was placed in the sun and kept close to 0° by a snow and water mixture. One-ml. samples were removed and analyzed for dissolved oxygen by a gasometric method (Scholander *et al.*, 1955). In 30 minutes the oxygen rose from 3.6 to 8.4 mm^3/cc. Soon after this, bubbles formed indicating supersaturation of the dissolved oxygen. Thus the sample of *Fucus* was still able to photosynthesize actively, even directly after being unnaturally frozen during the summer.

Fortunately it was possible to return to the same spot in March and check on the winter condition of the *Fucus*. The ice was many feet thick along the shore. In places tidal stresses had buckled it and the projecting sheets contained some of the algae frozen into the ice when it formed. This *Fucus* was fully exposed to the air temperatures which, during the brief visit, were as low as − 26° C. Earlier in the winter they had dropped to − 40° C. or lower.

Pieces of ice with *Fucus* in them were chipped free and thawed. The melted water contained only about 0.3 per cent salt. The alga was again checked for photosynthesis with the same positive result. The winter respiration rate was found to be close to that in the summer. There was no sign of a large oxygen debt from the long period in the ice. The *Fucus* is apparently ready to start active growth again in the spring where it left off in the fall.

These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Arctic Institute of North America. Reproduction in whole or in part is permitted for any purpose of the United States Government. I am deeply indebted to Dr. Per Scholander for advice and encouragement. My wife has been of considerable assistance both in the field and laboratory.

SUMMARY

1. As much as 80 per cent of the water in intertidal marine algae is frozen when exposed to the low air temperatures that regularly occur in nature.

2. The same species may lose 90 per cent of their water by ordinary drying during tidal exposure.

3. Metabolism is greatly depressed in both the frozen and dried states.

4. The ability to withstand drying may be related to freezing hardiness.

5. Some extreme conditions in the Arctic are described. *Fucus* spends many months frozen into the sea ice at temperatures down to −40° C., yet it is capable of photosynthesis immediately upon being thawed out.

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SOUND PRODUCTION IN THE SPINY LOBSTER PANULIRUS ARGUS (LATREILLE)[1, 2]

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That crustaceans contribute significantly to marine sound is well-known. A list of marine sound producers prepared by the United States National Museum as early as 1942 included members of 17 crustacean families (Fish, 1954). The significance of crustacean sounds to crustacean behavior and to the behavior of other marine animals is, however, largely unknown. There is some evidence that spiny lobsters can detect the vibrations of the sounds they produce (Lindberg, 1955; Dijkgraaf, 1955). Cohen (1955) rejects the statocysts of the lobster, *Homarus americanus,* as auditory organs in the sense of responding to pressure waves in the water, but suggests that statocyst vibration receptors may detect substrate vibrations accompanying sounds.

During June, July and August of 1956, while a guest of the Lerner Marine Laboratory of the American Museum of Natural History, I studied the acoustical behavior of the West Indian spiny lobster, *Panulirus argus.* A study was made of the anatomy of its sound-producing mechanism, and recordings were taken of the sounds produced by this species under various conditions. Through direct observation and motion pictures, data were obtained on the behavior of this spiny lobster in relation to sound production. The study was performed on North Bimini Island, the site of the Lerner Marine Laboratory.

Listening and recording equipment used in the investigation consisted of two Rochelle salt hydrophones, one an AX–58–C, the other undesignated, a Woods Hole Suitcase amplifier or a modified Heathkit amplifier Model A–7C, and an Ekotape tape recorder Model 205. Recordings were made at speeds of 3¾ and 7½ in./sec., and were analyzed on a Vibralyzer vibration frequency analyzer at the Woods Hole Oceanographic Institution. Sound-generating equipment employed in the experiments consisted of a Hewlett-Packard audio oscillator Model LAJ or the Ekotape tape recorder, a Craftsman C550 amplifier, and a QBG transducer.

The Mechanism of Sound Production

Stridulation by spiny lobsters (*Palinuridae*) has been described by a number of authors (Mobius, 1867; Kent, 1877; Goode, 1878; Parker, 1878; Heldt, 1929; Parker and Haswell, 1940, p. 455; MacGinitie and MacGinitie, 1949, p. 282; Dijkgraaf, 1955; Lindberg, 1955) and reference to it is found in classical literature (Yonge, 1854, p. 537). The characteristic rasp of palinurids is produced by an

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intricate stridulatory mechanism which has been partially described by Parker (1878) and Dijkgraaf (1955) in a Mediterranean species (*Palinurus vulgaris*). The following description is based on the mechanism as it exists in both males and females of *Panulirus argus,* and adds detail to descriptions previously published of other species.

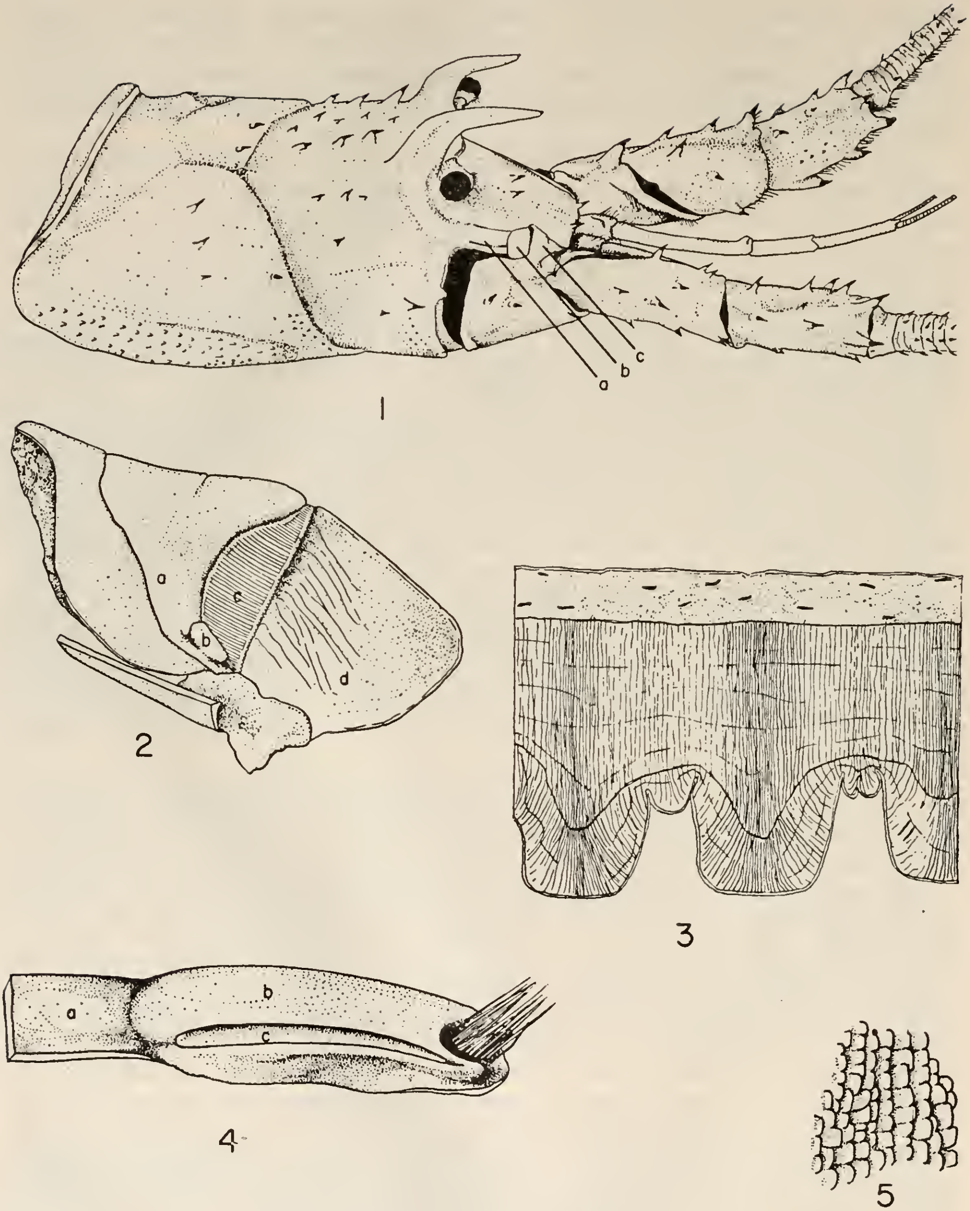
The acoustic mechanism of *P. argus* consists in part of a toothed ridge, orange-colored in life, rising from the surface of the carapace medial to each antennal base and extending anteriorly from beneath each stalked eye (Fig. 1a). A medial process of the basal segment of each antenna fits over the corresponding ridge; as an antenna is raised, a chitinous longitudinally-ridged membrane, the stridulatory membrane (Fig. 2c), forming part of the undersurface of the medial process, moves proximally over the toothed ridge, in the manner described by Dijkgraaf (1955) for *P. vulgaris,* so that a sound may be produced; the sound varies with the rate and force of raising of the antenna.

That a sound is produced only when the stridulatory membrane moves proximally over the toothed ridge—that is, when the antennae are raised—is due to a combination of factors. The medial process of the antenna is itself jointed, the process being divided into a basal flange (Figs. 1c, 2a) firmly united with the basal antennal segment, and a terminal portion (Figs. 1b, 2d) joined to the posterior edge of the flange. This terminal portion, which Dijkgraaf describes as being a freely-projecting skin-fold in *P. vulgaris,* is covered on its upper surface in *P. argus* with typical exoskeleton. Most of its under surface is formed by a soft chitinous membrane covered with a dense mat of minute setae, the setae becoming sparse along the leading edge of the stridulatory membrane. The under surface of the joint between the basal flange and the terminal portion is the site of the stridulatory membrane.

As a result of the arrangement described, when an antenna is suddenly swept back, the jointed edge of the basal flange bears down on the stridulatory membrane so that the latter is forced to rub proximally over the toothed, orange ridge and a sound is created. Lowering of the antenna relieves pressure on the membrane.

The second factor which determines the effective direction of antennal movement in producing sound is the presence on the portion of the orange ridge over which the stridulatory membrane moves of anteriorly projecting microscopic teeth (Fig. 5) against which the stridulatory membrane is forced when the antennae are swept back. These minute teeth are arranged in shingle-like fashion, teeth of adjacent rows alternating with each other, and the teeth of one row appearing to project from beneath those of the row behind. Anteriorly on the toothed ridge, the leading edges of the teeth form an angle of approximately 60 degrees with the surface of the ridge; in the posterior third of the stridulatory portion of the ridge, the edges are more nearly vertical. Anteriorly the edges of the teeth are slightly serrated; posteriorly they are smooth. In a narrow transition zone (transverse groove between a and b in Fig. 4) between the stridulatory (Fig. 4b) and non-stridulatory (Fig. 4a) parts of the ridge, the teeth give way to blunter microscopic projections, irregularly distributed, which are characteristic of the general carapace.

The presence of the teeth lends to the stridulatory portion of the orange ridge a dull appearance when the ridge is dry; the posterior portion of the ridge, from which the teeth are lacking, possesses a shiny surface when dry. The presence of the teeth can be detected by drawing the tip of the finger posteriorly along the



Stridulatory mechanism of *Panulirus argus.*

FIGURE 1: dorso-lateral view of head region; a) toothed portion of orange ridge, b) terminal portion of medial antennal process, c) basal flange. × 1.3.

FIGURE 2: under surface of medial process of right antenna; a) basal flange, b) guiding knob, c) stridulatory membrane, d) terminal portion. × 11.

FIGURE 3: cross-section of stridulatory membrane. × 650.

FIGURE 4: lateral view of right orange ridge; a) smooth portion, b) toothed portion, c) groove receiving guiding knob. × 7.

FIGURE 5: shingle-like teeth of dorsal surface of right orange ridge (anterior to the right). × 500.

toothed portion of the orange ridge. No difference to the touch between tooth-bearing and toothless portions of the orange ridge can be detected when the finger tip is drawn anteriorly.

The region of transition between the toothed and toothless regions of the ridge is marked by a shallow transverse groove (Fig. 4) which is the posterior limit reached by the stridulatory membrane when an antenna is raised. The action of the stridulatory membrane is guided by the presence of a small knob (Fig. 2b) projecting from the ventrolateral surface of the basal flange adjacent to the lateral end of the stridulatory membrane; the knob during raising and lowering of the antenna runs in a well-defined groove (Fig. 4c) on the lateral surface of the orange ridge. This groove extends as far posteriorly as the transverse groove already described. Behind the transverse groove, the orange ridge gradually flattens to the contour of the general carapace.

That the mat of short setae covering most of the under surface of the terminal portion of the medial antennal process becomes very sparse adjacent to the proximal edge of the stridulatory membrane allows one to observe that the surface from which the setae arise bears a polygonal, usually hexagonal, configuration. In this area individual setae spring from individual polygons; elsewhere, two to several setae arise from each polygon, and the dense mat of setae resulting obscures the underlying membrane.

Parker (1878) has figured a cross-section of the ridges of the stridulatory membrane in *P. vulgaris*. In *P. argus,* this membrane is constructed as follows (Fig. 3): there is a basal stratified layer of squamous epithelial cells. From this layer outward, the membrane is vertically striated to the level of the grooves between ridges, in a pattern which suggests that the major portion of the membrane is comprised of many fused setal processes. The ridges themselves comprise approximately ⅖ of the thickness of the membrane, and within the ridges, the striations radiate outward to the surface of each ridge. The whole surface of the stridulatory membrane is covered with a thin cuticular layer following the contour of the membrane. In surface view, the ridges are sculptured in a finely polygonal, usually hexagonal, pattern, the size of the polygons decreasing in the direction of the basal flange. Within the grooves between ridges the cuticular border is somewhat folded and the appearance of surface granulation of the ridges is absent. The stridulatory membrane is somewhat flexible, the relatively stiff ridges yielding a rasping sound when the tip of a dissecting needle is drawn across them.

The stridulatory membrane, as seen in cross-section, is also striated horizontally, in a pattern reminiscent of growth lines in skeletal parts of other animals (*e.g.,* tooth enamel, fish scales and otoliths). These horizontal lines continue into the ridges where they are curved in the contour of the ridges.

If the stridulatory membrane and the terminal portion of the medial antennal process are removed, raising of the antenna by hand produces only a slight squeaking sound as the joint edge of the basal flange bears directly on the toothed part of the orange ridge. The spiny lobster itself produces no sound on raising of an antenna so treated, as Parker (1878) also observed.

A specimen of *P. argus* which shed in one of the cement pools of the Lerner Marine Laboratory during the night of August 11–12, 1956, stridulated as usual in the morning, although the general exoskeleton was still soft. No animal from which the stridulatory mechanism had been removed shed during the summer, so that regeneration of the mechanism cannot be stated as fact.

The Sounds of Panulirus argus

The observation of Kent (1877), questioned by Goode (1878), that a shrill squeaking sound is produced by the spiny lobster (*Palinurus quadricornis*) by rubbing together of abdominal segments was probably accurate. During abdominal contractions, after *P. argus* is taken from the water, the abdomen is at times held tightly for a few moments under the cephalothorax, and a rather intense vibration is felt throughout the hand-held animal. At such times a squeaking sound, higher pitched than any antennal noise, may occasionally be heard. The body vibration accompanying this action is similar to that sometimes produced by the lobster, *Homarus americanus,* freshly removed from a trap or tank; while the latter does not produce an audible sound, vibration is at times so intense that an inexperienced person may drop the animal. This matter requires further study.

The antennal sounds of *P. argus* most frequently heard are either a rasp or a slow rattle of longer duration. The slow rattle, recorded during my study only when several animals were confined together in a live car, is seen after vibration analysis to consist of 5 or 6 pulses of sound spanning approximately .5 to 3.3 kc., the rattle lasting about ¼ second. The pulses are produced at an average rate of 27/second, varying in several cases from 24 to 31/second. The greatest intensity of each of these pulses lies at approximately .6 kc.

The antennal rasp, which usually accompanies abdominal contractions when a specimen is held in the hand in air or water, is a single burst of sound in which the individual pulses of sound cannot be distinguished. The sound lasts slightly over .1 second, and spans frequencies from below .04 kc. to approximately 9 kc. The zones of greatest intensity lie at .8 kc. and in a rather broad band between approximately 2.5 and 4.7 kc.

As a hand-held animal slows or ceases its abdominal contractions, the antennae may be swept back alternately rather than together. In sounds created by this action, individual pulses can be distinguished on vibration analysis. They are produced at rates varying between 56 and 133/second, with intensity peaks at the levels of the antennal rasp.

The rasp of *P. argus* can be roughly duplicated through moving the antennae upward by hand. The vibration analyses of sounds thus produced are like those of the rasp, except that instead of a single burst of sound there are from four to several bursts at somewhat irregular intervals; the upper peak intensity is more diffuse than in the normally produced rasp, and the lower peak (.8 kc.) of the normally produced sound is lacking. It has not been possible by this method to duplicate the slow rattle recorded from confined animals undisturbed by the observer.

Sound Production and the Behavior of Panulirus argus

That the production of sound by the spiny lobster is a response to definite stimuli is indicated by the observations of Lindberg (1955) and Dijkgraaf (1955). The antennae of *P. argus* are frequently moved without the production of sound, even when the basal segment of the antenna is involved. Aquarium-confined spiny lobsters trained to accept food offered by hand from above during the summer of 1956 characteristically raised themselves on their anterior legs, reaching their antennae up toward the proffered food. At such times, no sound was produced. Similarly, observations on live-car confined animals disclosed no antennal sounds produced during listening by hydrophone at times when the majority of animals were moving their antennae.

Although conditions surrounding production of the slow rattle are obscure, the sound bursts characteristic of the rasp frequently accompany strong, rapid abdominal contractions which are characteristic of the hand-held or net-captured animal. During these contractions, whether the restrained *P. argus* is struggling in the water or in air, the antennae are held back over the body and each forward thrust of the rapidly and forcefully contracting abdomen is accompanied by a brief rasp as the stridulatory membrane rubs over the orange ridge. Sounds intermediate between the slow rattle and the rasp are produced by antennal movements in the absence of abdominal contractions; although these intermediate sounds are difficult to distinguish by ear from the rasp, vibration analysis distinguishes them.

It is evident that the sounds produced by *P. argus* and the behavior which they accompany differ with circumstances. Dijkgraaf describes production of the rasp during struggling between two individuals of *P. vulgaris*. Circumstances under which the rasp is produced in *P. argus* (grasping in the hand, capturing in a net, injury) suggest that this sound is related to defensive behavior. This relationship is further emphasized by the fact that during production of the rasp, the spiny antennae are held over the back, tightly depressed against the carapace or against the grasping hand. During abdominal contractions, the forward-directed spines of the carapace are driven against the hand, and the hand is driven against the backward-directed spines of the antennae; blood is frequently drawn. At the same time, the abdominal contractions provide an efficient scissor-like action through the sharp edges of the abdominal exoskeleton, and the fingers caught within the scissors may be cut. Presumably the action described would also be performed against natural captors in the sea.

Gradual subsidence of abdominal contractions and of antennal sound by the hand-held animal will take place. A sudden movement of the hand will, however, re-initiate the whole process, and again a gradual subsidence, usually through a period of alternate antennal stridulations, will occur. The response gradually diminishes.

At the Lerner Marine Laboratory, up to several dozen spiny lobsters are confined at a time in a live car of screen and boards adjacent to the Laboratory dock in Bimini Harbor. When a hydrophone was lowered into this live car and left hanging in it during the daytime, the slow rattle already described was frequently heard. The individual pulses of the slow rattle, probably produced as the stridulatory membrane skips along the orange ridge, can be detected. The sound does not necessarily accompany marked activity of the animals to judge from visual observations, and is not recorded from a highly active animal. This slow rattle seems to be more in the nature of a conversational sound, as compared with implications of argument suggested by circumstances under which the rasp is produced.

The slow rattle could not be heard with the equipment employed 30 feet from the live car, although the rasp was clear over this distance underwater.

That the slow rattle is not produced by accidental movements of the antennae is suggested by the following observations: if the spiny lobsters in the live car were stirred up with an oar neither the slow rattle nor the rasp was heard, and after this procedure it was some time before the slow rattle was heard again; nearly constant antennal movements by isolated animals moving about in glass aquaria did not result in production of any sound, nor was the slow rattle recorded from any isolated animal; the slow rattle was recorded only during daylight, not at night when the spiny lobsters were moving about the live car; if an animal were suddenly clamped to the bottom with the edge of a dip net, only the rasp was heard to accompany the animal's activity, never the slow rattle which might have been anticipated if it were an accidental sound.

During the daytime, undisturbed *P. argus* tended to remain heaped in the corners of the live car; at night the animals were more obviously active, moving about the screen forming the sides of the car. Yet the slow rattle was heard only during the daytime. Thus, during 1½ hours of listening between 0900 and 1100 the morning of 21 June, the slow rattle was heard 76 times, whereas it was not heard at all during comparable time between 1950 and 2130 the evening of 20 June under an approximately ⅚ full moon. These observations were made when the live car contained between two and three dozen animals.

The stridulatory mechanism is best rendered inoperative by bilateral removal of the medial process of the antenna, at the junction of the basal flange and basal segment of the antenna. It is difficult effectively to remove the stridulatory membrane without removing the whole medial process, since even a remnant of the ridges will produce considerable sound.

A male spiny lobster with the stridulatory mechanism bilaterally removed was confined in an aquarium with a normal male of similar size during July of 1956. The normal animal was the dominant individual, frequently approaching the operated animal head-on with extended anterior walking legs and slightly raised antennae, walking forward as the operated animal retreated to a corner. At such times, no sounds were heard.

Numerous attempts to influence the behavior of spiny lobsters by playing recordings of the rasp and of the slow rattle into the live car and into aquaria containing individual specimens were unsuccessful, nor did recorded sounds of spiny lobsters played underwater noticeably alter the distribution of various fishes confined with them. The same was true if pulsating signals generated with the audio oscillator set at 17 to 40 cps. (Moulton, 1956) were played into the water.

Discussion

The production of two characteristic sounds by *Panulirus argus* and the behavior which the sounds accompany, parallel a situation occurring among fishes in sea robins (*Prionotus* spp.) which when free during the breeding season produce a staccato call, but which when disturbed in various ways produce a vibrant grunt of other frequency characteristics (Moulton, 1956). Like the staccato call of the sea robins, the slow rattle of the crayfish has not been recorded from hand-held or otherwise disturbed specimens. As the grunt and the staccato call of the sea robins relate to two different patterns of behavior, thus the rasp and the slow rattle of the spiny lobster, *P. argus,* do also.

The significance of their sound production to the survival of spiny lobsters is unknown. Lindberg (1955, pp. 178–179) observed that *P. interruptus* did not stridulate unless it and an attacking fish touched each other. Since the sounds produced by *P. argus* span the frequency sensitivity of various fishes which have been tested (Kleerekoper and Chagnon, 1954), it is not impossible that a combination of sound production and injurious strokes of the abdomen may combine as a deterrent to predators. Unfortunately, information is lacking on this point. My own observations concur with those of Lindberg in denying an obvious effect of the rasp on fish behavior. A mutilated specimen of *P. argus* passing, while vigorously rasping, through approximately 25 feet of clear water at Bimini on the west side of Turtle Rocks, did not immediately nor during several minutes of observation through glass panels at the surface attract any of the numerous reef fishes feeding within the zone of observation, nor did visible fishes which might be anticipated to feed on injured spiny lobster noticeably alter their behavior.

Dijkgraaf's (1955) observation that stridulation could be induced in a highly excited *P. vulgaris* by imitating the rasp adjacent to the aquarium is compatible with Lindberg's observation that *P. interruptus* moved away from other animals forced to stridulate nearby but out of sight. Lindberg observed that stridulation occurred only upon impending conflict and thus considers the sound "a threat rather than an alarm." Several *P. argus* contained in aquaria at Bimini moved rapidly away from a net or grasping hand, but did not stridulate until grasped or captured in the net—that is, the rasp was not necessarily produced during rather violent escape maneuvers.

The failure of fishes to respond to the sounds of *P. argus* is consistent with previous observations on the responses of free fishes to sound generally (Moulton and Backus, 1955; Moore and Newman, 1956). Since, however, several kinds of fishes will respond initially by quickened swimming movements—startle reactions—to sounds which later will fail to affect their behavior, it is possible that a sudden rasp by a mouth-held palinurid might cause a preying fish to release its hold and thus its prey, particularly if the sound were accompanied by strong abdominal contractions. This is to suggest that the rasp may act as a double assurance mechanism.

Vibration analyses of various fish sounds recorded from identified species at Woods Hole and Bimini indicate that sounds produced by several kinds of fishes under duress of various types are more like the rasp of the spiny lobster than like the slow rattle of undisturbed *P. argus* or than like the staccato call of free sea robins during the breeding season. The burst of sound characterizing the spiny lobster rasp also characterizes the sounds produced, during handling, by sea robins (*Prionotus* spp.), grouper and hind (*Epinephalus* spp.), angelfish (*Pomacanthus* spp. and *Angelichthyes ciliaris*), squirrelfish (*Holocentrus ascensionis*), triggerfish (*Balistes vetula* and *Melichthyes piceus*), swellfish (*Spheroides* spp.), porcupinefish (*Diodon hystrix*), a jack (*Caranx hippos*), and grunts (*Haemulon* spp.)—defensive behavior of all of these includes production of sounds lacking sharply defined individual pulses within the limits of the analyses. Thus sounds produced in a variety of ways—by stridulation of skeletal parts, by muscle contraction against an air bladder or adjacent tissue, by pounding of pectoral fins against the body—

but under similar circumstances, present similar, although not identical, vibration analyses. These observations, combined with observations on the slow rattle of *P. argus* and the staccato call of sea robins, suggest that while sounds produced by undisturbed marine fishes and crustaceans are likely to be sounds comprised of individual pulses of brief duration, sounds of marine fishes and crustaceans under duress are more likely to be bursts of sound of longer duration in which individual pulses are obscured or absent.

Another category of marine sound, comparable in its vibration analyses to sounds produced under duress by fishes and to the rasp of the spiny lobster, is the feeding noise of such fishes as wrasses (*Labridae*), porcupinefish, swellfish, triggerfish, and angelfish. If sound bursts comprise threats or alarms, as conditions of their production would suggest, noisy eating by these organisms may serve to repel rather than to attract predators.

Exceptional in producing sounds of sharp individual pulses during defensive behavior are representatives of two crustacean groups in the Bimini area, the stomatopod (*Gonodactylus oerstedi*) and several kinds of snapping shrimp (*Alpheidae*), especially *Alpheus armatus* and *Synalpheus* spp. (Pearse, 1950). Although produced in a different way, the sound of the mantis shrimp is like that of the snapping shrimp (Goode, 1878; Johnson, Everest and Young, 1947; Fish, 1954), and their vibration analyses are indistiguishable. Both are either burrowers or symbionts of organisms providing highly protected situations (Pearse, 1950; Townsley, 1953; Banner, 1953; Clarke, 1955). Further, the sounds produced are the by-products of accompanying movements which have a distinctly protective value—the decisive closure of the snapping shrimp large chela with its accompanying squirt of water (Schmitt, 1931, p. 192; Johnson *et al.,* 1947) and the stinging extension of the raptorial appendage of the mantis shrimp.

That some sounds of marine arthropods are other than accidental is, however, clearly indicated by the intricate stridulatory mechanism found among the spiny lobsters (Palinuridae). The usefulness of the sounds produced, as of the sounds produced by many kinds of fishes, is yet to be determined.

I am indebted for constructive criticism to Mr. William Schevill who read the manuscript of this paper, and for careful execution of the drawings to Mr. Ernest R. Powell, an undergraduate of Bowdoin College.

SUMMARY

1. The intricate stridulatory mechanism of the West Indian spiny lobster, *Panulirus argus,* and the sounds it produces are described. The sounds are related to various patterns of behavior.

2. On the basis of behavioral evidence it is suggested that a slow rattle is characteristic of spiny lobsters when contained in groups, and that a rasp is a usual component of defensive behavior.

3. The characteristics of the sounds of *P. argus* are compared to those of other marine sounds of biological origin. On the basis of this comparison, an attempt is made to generalize (a) the type of sound which accompanies defensive behavior of marine fishes and crustaceans, and (b) the type of sound stemming from marine fishes and crustaceans when undisturbed.

4. It is concluded that the intricacy of the sound-producing mechanism of *P. argus,* and of other palinurids, justifies a conclusion of a significance of sound to the biology of spiny lobsters. While certain suggestions of a possible value of the rasp to survival of spiny lobsters are presented, a consistent effect of the rasp on the behavior of other spiny lobsters and on predator organisms has yet to be demonstrated.

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ISOLATION AND ASSAY OF THE NEMATOCYST TOXIN OF METRIDIUM SENILE FIMBRIATUM

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The study of the immune mechanisms of marine invertebrates is inhibited by our lack of knowledge of the infectious diseases of these organisms. However, the many commensal relationships which exist between a variety of organisms and members of the Phylum Coelenterata suggest that a study of induced immunity to nematocyst toxins would at least yield information pertinent to the development of an understanding of antitoxic immunity in marine invertebrates.

There have been a number of attempts at the purification and description of these toxins (Cosmovici, 1925; Cantacuzène, 1926; Cantacuzène and Damboviceanu, 1934a, 1934b; Richet and Portier, 1936; Sonderhoff, 1936; Welsh, 1955). In all cases the material isolated represents extracts of the whole animal or some of its organs, *e.g.*, tentacles or acontia. As has been pointed out by Hyman (1940) in no case can it be certain that the material isolated is actually from nematocysts, the stinging capsules, and is not some toxic tissue component which normally does not play a role in the defensive or food gathering activities of the animal.

These present studies were carried out to develop a method of obtaining purified suspensions of nematocysts from sea anemones (*Actiniaria*) in order to obtain a toxic preparation which could be considered to be nematocystic in origin and could be used in studies on the antitoxic response of a variety of marine invertebrates.

Isolation of Nematocysts

The entire anemone was used as a source of nematocysts. Attempts to isolate nematocysts by enzymatic digestion of the surrounding tissues with pepsin, trypsin, ficin, and papain always resulted in damage to these structures, but the physical methods of separation described below have yielded suitable material. A number of variations in the method of preparation have been used, and the properties of the resulting materials have varied with the method. A few of these variations are included here since they illustrate species differences with regard to ease of purification and nature of the nematocysts, and these variations in method may be of help in similar investigations on other members of the phylum.

The anemones were first cleaned of adherent debris by placing them for a few days in a coarse wire mesh basket in an aquarium with running sea water. Approximately 500 grams, wet weight, of the animals were macerated in a Waring Blendor with 500 ml. of 1 *M* sucrose in sea water. An additional 500 ml. of this suspending medium was added, and the material was passed through a series of graded screens with openings of 1.168, 0.589, 0.295, and 0.147 mm. with the aid of suction. Tyler Standard Screens fastened to a Buchner funnel with masking tape were used. These screens remove the large particles of tissue from the suspension and allow the nematocysts to pass through, along with fine tissue debris, dissolved tissue components, and very fine sand. Filtrates from anemones, whose tissues contain symbiotic algae, bear these organisms as an additional contaminant. Upon centrifugation at 1000 rpm. for 15 minutes, the nematocysts were collected along with the sand, fine tissue debris, and algal cells if these were present. The sediment was washed free of dissolved tissue constituents by repeated re-suspension in the sucrose solution and re-centrifugation. This procedure also removed a considerable amount of the fine tissue debris. The nematocysts were purified further by differential centrifugations of 15 minutes and 15 seconds at top speed in a small International Clinical Centrifuge with a bucket head. The longer centrifugation left most of the fine tissue debris in suspension while the nematocysts were collected in the sediment. The shorter centrifugation left the majority of nematocysts in suspense but removed the sand. Five or six pairs of centrifugations were usually sufficient.

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Three criteria for the success of any method were employed, *i.e.*, purity of the suspensions, susceptibility of resulting nematocysts to artificial discharge, and toxicity of the material released on discharge. Particularly good results were obtained in the case of *Metridium senile fimbriatum*. Characteristics of these preparations are discussed below. However, the treatment of *Anthopleura xanthogramica* or *Anthopleura elegantissima* in this fashion resulted in unsuitable material. *A. xanthogramica* is infected with zooxanthellae which could only be removed by shaking the nematocyst suspensions with ether. Upon centrifugation the nematocysts were found in the sediment and the algal cells along with any remaining fine tissue debris stayed in the ether phase which had a gelatinous consistency. With this modification highly purified suspensions could be obtained, but the nematocysts could not be artificially discharged. While it proved possible to obtain, in protected, darkened areas, specimens of *A. elegantissima* which did not contain symbiotic algae, the nematocysts obtained from these animals were also not susceptible to artificial discharge.

Characteristics of Nematocyst Suspensions from Metridium senile fimbriatum

Approximately 0.5 gram, dry weight, of nematocysts was obtained from 500 grams, wet weight, of this species. The material was all but completely free of tissue debris and sand when examined microscopically. Continued differential centrifugation neither increased the per cent hexosamine content of 3.1–3.2% after hydrolysis nor decreased the total nitrogen content which was 10.2–10.4%. The per cent composition of different batches of nematocysts agreed within experimental error. A dried preparation could be obtained by washing suspensions with a solution of glycerine and distilled water, 1 : 1 by volume, followed by 95% ethanol and ether and drying in a desiccator.

The half-life of a purified nematocyst suspension appears to depend at least partly upon the osmotic pressure exerted by the suspending medium. When kept in the refrigerator the time required for discharge of one half of the nematocysts was 12 hours in 1 *M* sucrose in sea water, 7 days in 1 : 1 glycerine and distilled water, at least three months in 95% ethanol, and over 6 months for dried material. These observations are in agreement with those of Glaser and Sparrow (1909). The dried material would probably keep indefinitely (Weill, 1926). However, neither alcoholic suspensions nor dried material exhibits any toxicity. Both ether and alcohol effectively detoxify the nematocysts.

The spectrum of nematocyst types, cnidom, for *M. senile fimbriatum* has been described recently by Hand (1955). Table I gives the differential count for each of the types found in the suspensions. Some of the types have been divided into size categories which represent approximate mean dimensions. This variation in size with respect to a particular type makes a physical separation of one type from another extremely difficult. Until it can be determined that the toxin of one type is the same or different from the toxin of another type, it seems desirable to present such counts as a part of the description of the material whose toxicity is under investigation.

TABLE I

Differential count of nematocysts in suspensions from Metridium senile fimbriatum

Nematocyst type	Size (microns)	Counted	Per cent
Microbasic b-mastigophore	60 × 5	15	7.4
	30 × 4	6	2.9
	10 × 3	6	2.9
Microbasic p-mastigophore	20 × 3	2	1.0
	10 × 5	14	6.9
Microbasic amastigophore	70 × 7	10	4.9
	30 × 5	21	10.4
	10 × 4	8	3.9
Basitrich	20 × 4	20	9.8
	13 × 2	16	7.8
Atrich	24–47 × 7–15	2	1.0
Holotrich	13–23 × 4–6	7	3.4
Spirocysts	12–30 × 4	76	37.4
	Totals	203	99.7

NEMATOCYST TOXIN FROM METRIDIUM SENILE FIMBRIATUM

The purified suspensions contain 37–39% discharged nematocysts. The remainder can be artificially discharged by treatment with distilled water, methylene blue, weak acid, weak base, sodium thioglycolate, or sodium taurocholate. In order to obtain the maximum release of toxin, the nematocysts were placed in distilled water for 12 to 18 hours. After such a period all but approximately 1% are discharged. Such "normal" discharge was found to be just as effective a means of obtaining the toxin as grinding and extraction. Discharge released 21.8% of the dry weight of the nematocysts. Grinding with carborundum in a mortar and extraction with distilled water removed 21.7%. Discharge in a test tube may be followed with the naked eye. The tubes everted from the nematocysts become entangled and eventually form a slimy, cottony sediment.

Some information as to the chemical nature of the toxin has been obtained

(Phillips, 1956). Hydroxy-indoles were detected on paper chromatograms. In an attempt at isolation of these substances from large amounts of nematocysts, the content of 5-hydroxy-indoles was followed quantitatively, using the method of Mitoma *et al.* (1956) during the purification of the nematocysts. As the suspension became more and more free of tissue components, the level of 5-hydroxy-indoles dropped steadily. This would suggest that these substances are not a part of the toxin but instead represent a soluble tissue component.

Various marine invertebrates were tested for their susceptibility to extracts of the nematocysts of *Metridium senile fimbriatum.* The animal found to be the most convenient for assay purposes was *Littorina planaxis,* a small snail from the high intertidal zone. This animal normally has no contact with coelenterates of any sort, at least during its post-larval and adult stages. When placed upside down in sea water it rapidly rights itself and moves out of the water to a relatively dry place. It was found that the time required for this righting and withdrawal from the water could be prolonged by addition of the toxin and the length of inhibition is dependent upon the concentration of the toxin. With very high doses of the toxin the time was infinitely extended since there was a resulting death of the snail.

TABLE II

Per cent inhibition of the righting response of Littorina planaxis by distilled water extracts of the nematocysts of Metridium senile fimbriatum

Dose, micrograms	Time, minutes				Hours				Days			
	5	10	20	40	1	2	4	8	1	2	4	8
150.0	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	80%*
75.0	100	100	100	100	100	100	100	100	80	80	70	40**
37.5	100	100	100	100	100	100	100	100	70	30	30	0
18.75	100	100	100	100	100	90	80	50	0	0	0	0
9.375	100	90	80	80	80	80	40	20	0	0	0	0
7.5	100	100	90	30	20	0	0	0	0	0	0	0
0.75	100	80	40	20	0	0	0	0	0	0	0	0
0.00	70	60	40	0	0	0	0	0	0	0	0	0

* 8/10 dead.
** 4/10 dead.

The titrations of toxicity were carried out as follows: The various doses of toxin were prepared in 1 ml. of sea water and placed in flat bottomed tubes, 25 × 95 mm. Ten tubes of each dose were prepared so that ten snails per dose could be tested simultaneously. The snails were dried with a towel, and the water in the mantle cavities was removed by gentle pressure on their opercula with a towel-covered probe. They were then dropped into the toxin and shaken so that the snails were upside down and the cup of the shell was filled with toxin. One milliliter of the toxin dilution was insufficient to cover the snail, so that the amount of diluted toxin actually involved in the test was the amount which was contained in the cup of the shell and was ultimately drawn into the mantle cavity. The time at which the snails were first dropped into the diluted toxin was noted, and the time at which they righted themselves was noted. Table II shows the results obtained with various doses of the toxin. The dose is expressed in micrograms dry weight of toxin contained in 1 ml. of sea water. For convenience, 1½ hours was taken as the period of observation in subsequent titrations.

Since *Littorina* is exposed to fresh water in the form of rain at not infrequent intervals, it seemed unlikely that the righting response would be affected by dilution of the sea water with distilled water. However, since the toxin was obtained by discharge of the nematocysts in distilled water, the effect of the dilution of the sea water by the addition of toxin was determined. No effect due to dilution could be found over the range used in the test.

The snails employed in the titrations show a considerable variation in size. On the basis of body weight, including shell, they vary from 1.15 to 0.33 grams. Yet their response to the toxin did not appear to be correlated with body weight. In order to explain this observation a group of snails was weighed independently before and after the removal of the fluid from the mantle cavity. In this way the volume and weight of the effective dose received by the snails of various weights were determined. It was found that the dose received by each snail is proportional to its body weight. The volume of sea water that contains the toxin and is drawn into the mantle cavity varies from 0.10 to 0.01 ml., depending on the size of the snail. This means that the effective dose of toxin is ¹⁄₁₀ to ¹⁄₁₀₀ of the amount present in the milliliter of toxin dilution.

TABLE III

Per cent inhibition of the righting response of Littorina planaxis by toxin of Metridium senile fimbriatum obtained by discharge of nematocysts in two different media

	Toxin obtained by discharge in:	
Dose (micrograms/ml. of sea water)	Distilled water % Inhibited	Sodium thioglycolate % Inhibited
30.0	90	100
15.0	80	50
7.5	20	40
3.75	10	0
1.875	0	0
ED_{50}	12	11.5
$f_{ED_{50}}$	1.52	1.75
95% confidence limits of ED_{50}	7.9–18.2	6.5–20.4

The toxin was assayed using five doses and ten snails per dose. The titrations were terminated after 1½ hours. Since there was a possibility that the toxin was sensitive to oxygen, toxin obtained by discharge of the nematocysts in 0.05% sodium thioglycolate was compared with toxin obtained by discharge in distilled water. The method of Litchfield and Wilcoxon (1949) was used to obtain: (a) the median effective dose, ED_{50}; and (b) the factor, $f_{ED_{50}}$, for obtaining the 95% confidence limits of the ED_{50}. The curves were found to be parallel within experimental error. Table III shows the results of these titrations and includes the parameters mentioned above. It does not appear that the toxin is particularly oxygen-labile.

SUMMARY

1. A method of obtaining purified suspensions of nematocysts has been developed, and a method of obtaining the toxin they contain has been described.

2. A method of assay, using inhibition of the righting response of *Littorina planaxis*, has been shown to be applicable for toxicity titrations.

3. Further work on the chemical and immunochemical characterization of the toxin is planned, as well as the use of the toxin for studies of antitoxic immunity in marine invertebrates.

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STUDIES ON THE LIFE-HISTORY OF ALLOCREADIUM ALLONEOTENICUM SP. NOV. (ALLOCREADIIDAE–TREMATODA)

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The systematics of the trematode genus *Allocreadium* has been confused because of studies which reported two cercarial types from members of the genus. The cercariae which have been reported are not only morphologically dissimilar, but the molluscan hosts include both sphaeriid clams (Looss, 1894; Dollfus, 1949; and Peters, 1957) and prosobranch snails (Seitner, 1951).

Looss (1894) and Dollfus (1949) described an ophthalmoxiphidiocercaria developing from rediae in sphaeriid bivalves as the cercarial stage of the type species *A. isoporum* (Looss 1894). Peters (1957) also described a similar ophthalmoxiphidiocercaria from rediae developing in sphaeriid bivalves as the cercarial stage of *A. neotenicum* Peters 1956. These three authors did not demonstrate the life cycles of the parasites experimentally but based their conclusions on morphological similarities of the cercariae and adults. The ecological evidence presented in each case supported their conclusions.

Seitner (1951) described the larval forms of *A. ictaluri* Pearse 1924, from *Pleurocera acuta* (Say), a prosobranch gastropod. In this case, the cercaria was described as a gymnocephalous biocellate form, bearing setae in symmetrically arranged papillae on the body and tail. Seitner pointed out that it is extremely unlikely that species in the same genus would be morphologically different and that they would have such widely different molluscan hosts. Since Seitner's work was supported by experimental evidence he correctly regarded the earlier conclusions of Looss and Dollfus as inconclusive. Peters (1957) pointed out, however, that there are several reasons to question whether the cercaria described by Seitner is actually the larva of *A. ictaluri*. Peters further showed that morphological and ecological data tended to prove that Seitner was probably dealing with the larval stages of *Skrjabinopsolus manteri* (Cable 1952), a lepocreadioid, instead of *A. ictaluri*.

Mathias (1937) reported on the life-history of *Allocreadium angusticolle* (Hausmann) but this trematode has since been placed in the genus *Coitocaecum* by Dollfus (1949).

Evidence from controlled experiments in the present study supports the morphological and ecological observations of Looss, Dollfus and Peters, since the molluscan hosts are sphaeriid clams which liberate ophthalmoxiphidiocercariae. These penetrate into caddis fly larvae and become precociously mature in the haemocoel of these hosts.

METHODS AND MATERIALS

Materials used in this study were obtained from the clam, *Pisidium abditum* Haldeman, and from caddis fly larvae belonging to the genus *Limnéphilus* collected at two localities near Falmouth on Cape Cod, Mass. One collecting area was a spring-fed pond draining into the Coonamessett River just off Sandwich Road and the other was an extensive cranberry-ditch area along the Quashnet River just off Highway 28. Other caddis fly larvae and various beetle larvae and adult beetles occurring in the type localities were never infected in nature and were refractive to experimental infection. Living material, from both fingernail clams and caddis fly larvae, was used for the study of the excretory system and other morphological details.

A caddis fly larva harboring adult flukes (Fig. 1) could be determined by forcing the larva partially out of the case and observing the appendages and abdominal area for the presence of the eggs of the parasite. The infected larvae were characteristically darker brown than uninfected ones because of the presence of the eggs. If eggs were present in the larvae, at least one adult fluke was present. In a few cases, the fluke had degenerated so that they appeared as a blackish, internally amorphous mass which, however, still retained the characteristic external shape of the worm. Various degenerative stages of the flukes were observed to form an uninterrupted series so that it was evident that this was not an isolated or abnormal occurrence. These stages were more often found in caddis fly larvae harboring many flukes. (Some contained up to 25 worms.)

Worms and eggs were obtained by carefully detaching the head from the larvae, dissecting the last two segments from the abdomen and withdrawing the intestine, thus allowing space for the escape of the flukes from the haemocoel.

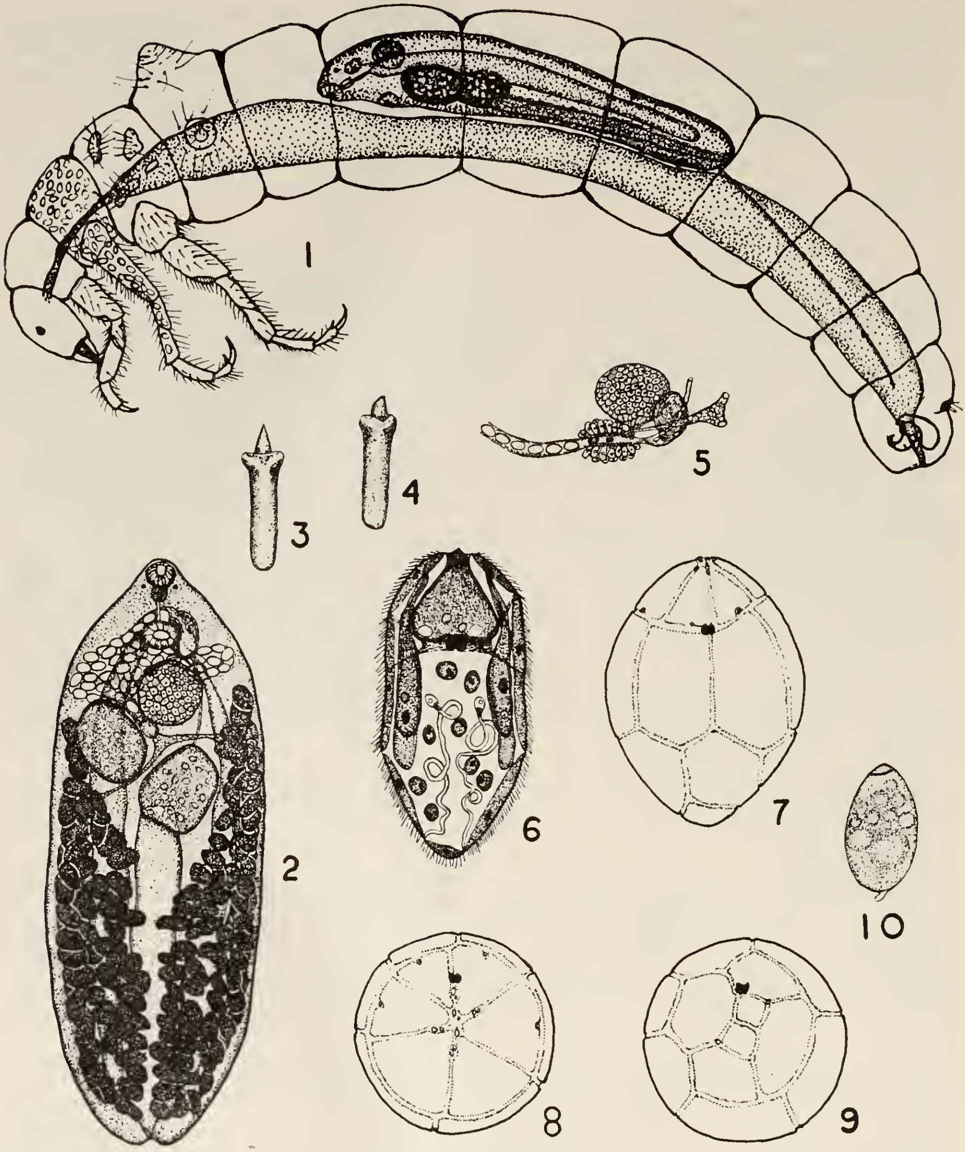
Specimens were fixed by squirting them into Gilson's fluid at 60° C. Whole mounts were stained with Semichon's aceto-carmine and Harris' haemotoxylin. Sections of uninfected and infected caddis fly larvae with worms *in situ* were stained with Delafield's haematoxylin.

Cercariae were studied alive with the aid of aqueous vital stains such as neutral red, orange G, brilliant cresyl blue, and Nile blue sulphate and also as fixed and stained specimens.

Two methods were used to obtain miracidia for study. One was to wash out as many eggs as possible from dissected material, then to remove the remains of the larvae and wash the eggs. Such "clean" eggs did not hatch even when filtered water from finger bowls containing an abundance of dead leaves and other organic material was substituted for stream water. When numerous miracidia could be observed actively moving within the eggs, small snails, *Aplexa hypnorum* (L.), from Coonamessett pool were added. The snails readily ingested the eggs and as eggs appeared in the feces of the snail, the miracidia began to hatch. This process was observed under the dissecting microscope. The other method of obtaining miracidia, which gave equal results as far as numbers of miracidia was concerned, was to dissect the caddis fly larvae but to leave the remains in the container with the eggs and to add filtered water rich in organic acids. This method had the disadvantage of encouraging bacterial and protozoan growth.

Miracidia obtained by these two methods were studied, alive by the use of vital stains, and also as fixed and stained specimens. For the study of the epidermal plates, the miracidia were impregnated using the silver nitrate method of Goodchild (1948) and mounted in glycerine jelly.

All measurements used in this study are in millimeters. Length is given first, followed by width.



Explanation of Plate I

Figure 1. *Limnéphilus* sp. (10 mm. in length); lateral view with *Allocreadium alloneotenicum* adult in haemocoel. Eggs shown only in middle thoracic segment. Entire figure diagrammatic.

Figure 2. Adult (3.5 × 1.3 mm.), ventral view.

Figure 3. Cercarial stylet (0.0238 mm. in length).

Figure 4. Worn stylet of adult (0.023 mm. in length).

Figure 5. Female complex, lateral view.

Figure 6. Miracidium (0.095 × 0.045 mm.), dorsal view.

Figure 7. Miracidial epidermal plates (0.061 × 0.044 mm.), dorsal view.

DESCRIPTIONS OF STAGES IN THE LIFE-CYCLE

Allocreadium alloneotenicum n. sp.: The specific name *alloneotenicum* ("another" neotenicum) was chosen because of the similarity in neotenous development to *A. neotenicum* Peters 1957.

Host: The caddis fly larva *Limnéphilus* sp.
Site: Haemocoel.
Incidence: 40 of 120 larvae (30%).
Type locality: Coonamessett River, Barnstable County, Cape Cod, Massachusetts, U. S. A.
Type specimens: A type and paratype will be deposited in the Helminthological Collection of the United States National Museum.

Adult (Fig. 2):

Body elliptical, 1.38–4.42 × 0.59–1.41 mm., thick but slightly flattened dorsoventrally, anterior end bluntly pointed; usually with posterior indentation at excretory pore; cuticle smooth. Well developed eyespots, located at level of posterior margin of oral sucker, present in all stages of development. Oral sucker subterminal 0.12–0.20 × 0.08–0.19 mm. (average 0.16 × 0.14 mm.). Stylet present (Fig. 4), imbedded in dorsal lip of oral sucker in relatively same position as in cercaria at an angle of 45°–60° from the longitudinal axis of the body. Ventral sucker 0.13–0.22 × 0.10–0.22 mm., (average 0.175 × 0.156 mm.), in anterior one-sixth or one-seventh of body. Prepharynx short, usually not evident but distinguishable in sections. Pharynx spherical, 0.06–0.11 mm. in diameter, posterio-dorsal to oral sucker. Esophagus as long as, to twice as long as, pharynx; intestinal bifurcation at level of anterior edge of ventral sucker, caeca simple, somewhat inflated, extending dorsally almost to posterior end of body. Excretory bladder elongate, sac-shaped with well defined lumen, extending anteriorly to middle of posterior testis. Excretory pore terminal within indentation at posterior end of body. Main excretory tubules extend anterio-laterad from bladder in a somewhat tortuous pattern, but without recurrent loop to level of mid-anterior testis where each receives an anterior and posterior secondary tubule. Each secondary tubule drains three groups of flame cells, exhibiting considerable variation both in numbers and position. Apparent flame cell formula is $2\,[(6+6+6)+(6+6+6)]$.

Ovary oval, 0.21–0.37 × 0.19–0.46 mm. (average 0.28 × 0.31 mm.), posterior to ventral sucker, sometimes overlapping the latter. Oviduct extends mediad a short distance, receives the common duct of receptaculum seminis and Lauer's canal (Fig. 5), then turns anteriad. Receptaculum seminis variable in shape, usually about one-fourth size of ovary. Sinuous Lauer's canal opens dorsally, posterio-median to ovary. Oviduct extends to form the oötype which is surrounded by a prominent Mehlis' gland; from oötype the uterus extends anteriorly composed of few loops, usually confined to area on right side of body bounded posteriorly by anterior testis and medially by ovary, but occasionally overlapping these struc-

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FIGURE 8. Miracidial epidermal plates (0.044 mm. in diameter), anterior view.
FIGURE 9. Miracidial epidermal plates (0.044 mm. in diameter), posterior view.
FIGURE 10. Egg (0.096 × 0.056 mm.), lateral view.

tures. Uterus sometimes with a loop or two anterior to the ovary on left side of body. Metraterm present, opening into genital pore. Shallow genital pore ventromedian, near level of pharynx.

Vitelline follicles extend posteriad from anterior edge of ovary, more or less confluent in post-testicular half of body on ventral side, reaching almost to posterior end of body. Right and left vitelline ducts unite to form the vitelline reservoir in the angular space between the testes and ovary.

Testes oval or irregular in outline, never lobed, subequal, anterior testis, 0.19–0.46 × 0.24–0.44 mm., (average 0.29 × 0.35 mm.); posterior testis, 0.19–0.51 × 0.19–0.59 mm., (average 0.31 × 0.39 mm.). Testes diagonal, contiguous or separated by a short distance, ventral in position and confined to anterior one half of body. Vasa efferentia arise on anterior margin of testes, proceed anteriorly and join at posterior end of cirrus sac to form a very short vas deferens which enters cirrus sac forming a vesicula seminalis. Cirrus sac, 0.09–0.22 × 0.12–0.34 mm. (average 0.14 × 0.22 mm.), median or submedian to left of mid-line, between pharynx and ventral sucker; vesicula seminalis convoluted, pars prostatica tubular, prostate cells numerous and well developed.

Eggs number up to 45, measuring 0.092–0.108 × 0.055–0.060 mm. (average 0.0975 × 0.0577 mm.) with a small antopercular knob (Fig. 10), shell thin, light golden brown.

Miracidium (Fig. 6):

When first laid, the eggs are segmented. Development of the eggs is extremely variable. Within 48 hours, motile miracidia can be observed in some eggs, in others movement of miracidia can not be observed for several days. Upon hatching, the miracidium swims in a slightly zig-zag path, rotating slowly. Miracidia positively phototactic, converging either on light side of shallow dish or on opposite side where light rays are concentrated after passage through the water. Body of miracidium either elongate or pear-shaped when swimming, terrebratorium sometimes protruded. Miracidia were infective to both laboratory-reared young *Pisidium abditum* and *Musculium partumeium* (Say). No attraction to clam hosts was observed but infections were present within 24 hours after clams were added to the container with miracidia.

In using the Goodchild (1948) modification of Lynch's (1933) silver nitrate method of delineating epidermal plates, it was found that if the 1.0% silver nitrate solution was warmed slightly, there was less distortion of the miracidium. Silver nitrate-treated miracidia measured 0.061 × 0.044 mm.

The epidermal cell formula is 6–6–4–2 (Figs. 7, 8, 9). The same formula was observed by Peters (1957) for *A. neotenicum*. The miracidia of both these neotenous forms are quite similar, agreeing closely in most respects. The anterior and second tiers consist of two ventro-lateral, two dorso-lateral and two lateral cells lined up essentially end-to-end. The four cells in the next tier are arranged with a ventro-lateral and a dorso-lateral cell on each side, while the posterior tier consists of a dorsal and a ventral cell. The conspicuous double eyespots are situated dorsally about one-third of the length from the anterior end in living miracidia. The nervous system surrounds the eyespots and sends branches laterally to sensory pores and posterior branches diagonally to the middle of the lateral ciliary plates in the second tier. The anterior fourth of the body is occupied by the apical gland which is without a stylet, and with 3–4 nuclei along its posterior margin. Granular structures representing the "penetration" glands of other authors are difficult to see clearly. They occupy most of the lateral portions of the miracidium. Observations of miracidial penetration clearly show four discrete unicellular glands. Two flame cells, usually not at same level, lie near the middle of the miracidium. Five to nine germinal cells are scattered in the center of the body posterior to the eyespots. Living miracidia measure 0.068–0.108 × 0.036–0.052 mm.

Miracidial penetration into the molluscan host has been observed by several workers, notably Thomas (1883) in *Fasciola hepatica,* Barlow (1925) in *Fasciolopsis buski,* Bennett (1936) in *Cotylophoron cotylophorum,* Rees (1940) in *Parorchis acanthus* and Goodchild (1948) in *Gorgodera amplicava.* Barlow suggested that the apical gland secreted an "erosive fluid" while Goodchild considered it to secrete an adhesive substance. Several authors have observed droplets of fluid at the pores of the "penetration" glands and have assigned penetration functions to the secretions from these glands. Bennett pointed out that as transformation of the miracidium took place, it gradually changed shape and a very thin cuticula was formed around the outside of the body, but he did not give its origin.

In order to study the process of pentration and transformation of the miracidium into the sporocysts, an excised gill from a small uninfected *P. abditum* was added to a drop of water containing several miracidia on a slide. The actively swimming miracidia came into contact with the gill many times before they started to penetrate into the gill tissue. No attraction to the tissue was evident. The process of penetration was observed under high magnification of a compound microscope.

When penetrating into the gill, initially there is a rotatory movement combined with extreme prolongations of the anterior end of the larva, followed by progressive swelling from anterior to posterior, which draws the miracidium into the tissue. After approximately 15 minutes, rotary movements cease and the miracidium begins a rhythmical contraction and elongation of the body. Four small distinct droplets of granular material (Fig. 11) are extruded from the pores of the "penetration" glands. These pores are not symmetrically arranged although they each open in the anterior space between the first tier of ciliary plates. On the left of the miradicium one pore opens in the space between the dorso-lateral plates, while the other opens between the dorso-lateral and the lateral plates. On the right side of the body, however, one of the pores opens between the two ventro-laterals and the other between the ventro-lateral and lateral plates. The droplets coalesce into two droplets (Fig. 12). At this time granular material lighter in color and more fluid in consistency begins to flow from pores on the terrebratorium draining the apical gland. This material appears to be histolytic in function since there is a progressive breakdown and liquefaction of the clam tissue anterior to the miracidium. As additional granular material is extruded from the pores of the "penetration glands," the droplets fuse into an apical cap covering the anterior end. It could not be clearly observed if the secretion of the apical gland was pushed ahead of the forming cap or if it mixed with the cap material. At least some of the apical gland secretion stays anterior to the apical cap. As the apical cap becomes more extensive (Fig. 13), the sporocyst begins to extend into it from the miracidial covering by way of the terrebratorium.

The process of sporocyst emergence (Figs. 14–18) requires over three hours. During this time the apical cap becomes increasingly thicker until it extends 0.015

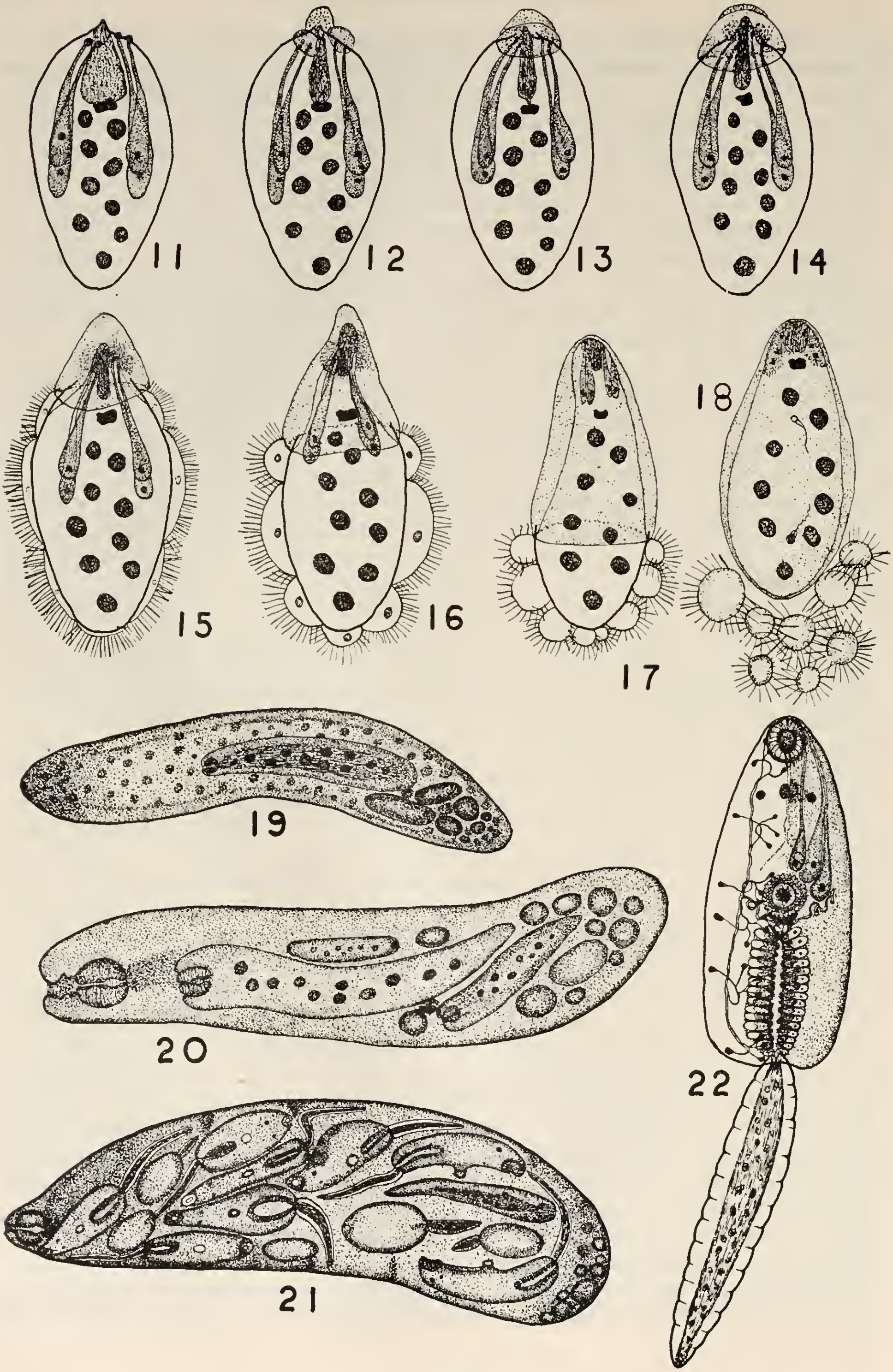
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PLATE II

mm. anterior to the miracidium. The sporocyst gradually emerges into the apical cap material from the miracidial covering by a rhythmical series of anterior extensions and expansions. It is always covered by either the miracidial covering or the cap material since the latter gradually extends more posteriorly. As the granular material extends posteriad it appears to force the epidermal plate cells ahead of it. The epidermal cells become quite evident, each with a well defined nucleus. When the cap material covers about three-fourths of the emerging sporocyst, the epidermal cells are almost spherical and their cilia are perpendicular to the cell membrane and are still actively beating. A similar appearance of epidermal plate cells was described by Thomas (1883) and by Barlow (1925). Three hours after the sporocyst begins to emerge, the cap material completely covers the body of the sporocyst. This cuticle is more evident on the sides of the sporocyst than it is on the ends, being twice as thick on the former as on the latter. As the cuticle is fully formed, the rounded epidermal cells break away and are moved about by their beating cilia.

The apical gland thus appears to secrete a histolytic substance which aids in penetration and the "penetration" glands do not seem to aid in penetration other than perhaps passively by filling the space eroded by the material from the apical gland. Since the "penetration" glands do secrete material which forms a cuticle for the sporocyst, it would be more correct to call these "cuticle-producing" glands and assign the penetration function to the apical gland.

Sporocysts:

Newly formed sporocysts measure 0.061–0.068 × 0.029–0.031 mm. Eyespots are still contiguous and the apical gland and cuticle-forming gland remnants are confined to the anterior end of the sporocysts.

Additional development of the sporocysts was followed mainly in infections in *P. abditum* but *M. partumeium* infections were followed for two weeks and development in the two clam hosts was parallel. Presumably the infection in *M. partumeium* will also develop to the cercarial stage, but limited numbers of small clams of this species did not allow further study of development.

Much of the variation observed in the following developmental stages is due to the fact that clams were left in containers with hatching miracidia to insure infection, and since miracidia continued to hatch for several weeks, superimposed infections were common.

Four days after infection, the smallest sporocyst observed measured 0.08 × 0.051 mm., developing in the gill of the clam. Two eyespots were present, one in the middle of the anterior end and one almost midlength on the side of the body. No evidence of a sucker was found. Nine developing germinal cells occupied most of

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EXPLANATION OF PLATE II.

FIGURES 11–14. Penetrating miracidium, showing secretions from "penetration" and apical glands and formation of apical cap (cilia omitted).

FIGURES 15–18. Formation of cuticle by gradual posterior progression of cap material forcing ciliated epidermal cells away from new sporocyst.

FIGURE 19. Sporocyst (0.42 × 0.082 mm.).

FIGURE 20. Mother redia (0.60 × 0.120 mm.).

FIGURE 21. Daughter redia (0.090 × 0.25 mm.).

FIGURE 22. Cercaria (body 0.341 × 0.15 mm., tail, contracted, 0.30 × 0.063 mm.), ventral view. Detail shown on the left and excretory system on the right of body.

the body space. The two flame cells were situated diagonally in the middle of the body.

Seven days after infection, clams yielded several sporocysts that measured 0.108–0.136 × 0.035–0.08 mm., with pigment sometimes diffuse but usually present as distinct eyespots, variable as to their location.

Eighteen days after infection, sporocysts measured 0.103–0.274 × 0.047–0.072 mm. In all sporocysts at this stage of development, eyespots were still present, and two flame cells were easily seen, one posterior and one anterior. Usually one germinal ball (sometimes two) had increased in size, and one was usually three or four times as large as the other germ balls.

By the 25th day the sporocysts were widely distributed in the tissues of the clam, being present in the gills, foot, digestive gland and mantle. These sporocysts ranged in size from 0.244–0.494 × 0.072–0.108 mm. At this stage eyespot pigment was usually diffuse or absent. An identifiable redia, with its conspicuous globular sucker, was usually present in each sporocyst. By the time the infection was 33 days old, rediae could be found in the tissues of the clam but their escape from sporocysts was not observed. Sporocysts (Fig. 19) increased very little in size beyond 0.50 × 0.12 mm.

At this stage, rediae surpassed the sporocysts in size, varying from 0.288 × 0.057 to 0.63 × 0.075 mm. The redial sucker is large, reaching 0.055 × 0.048 mm. The mother rediae (Fig. 20) are morphologically the same as the daughter rediae but their germinal cells give rise only to rediae and not to cercariae. Mother rediae usually contained several developing rediae, one of which was typically larger than the other.

In infections from 40 to 50 days old, sporocysts appeared to be absent while mother rediae with a maximum size of 0.73 × 0.094 mm. were still producing daughter rediae. There appeared to be only one generation of mother rediae, since daughter rediae began to differentiate identifiable cercariae when the infection was 50 days old.

Mature daughter rediae (Fig. 21) are elongate, thin-walled sacs without locomotory processes. The sucker is spherical, 0.04–0.057 mm.; the intestine reduced and inconspicuous. The birth pore is just anterior and lateral to the sucker, not clearly visible, but cercariae were observed escaping, one by one from the pore.

Twelve cercariae with definite eyespots were the maximum number observed in any redia. Combinations of developing cercariae with eyespots, and germinal masses numbered up to 18. Rediae containing eyed-cercariae measured 0.52–1.16 × 0.12–0.30 mm. (average size 0.87 × 0.20 mm.; average number of eyed-cercariae 7). Occasional rediae contained eyed-cercariae, germinal masses and daughter rediae. Rediae were also observed containing germ balls and daughter rediae. This would indicate that asexual multiplication is a continuous process and possibly continues for the life of the infection. Flame cells were four in number and appeared to be paired into two homologous systems, each with an anterior and a posterior flame cell. However, the ducts were difficult to distinguish.

Cercaria:

The cercaria is an ophthalmoxiphidiocercaria (Fig. 22), ellipsoidal in outline, from two to three times as long as wide, slightly depressed dorso-ventrally with unarmed cuticle. Anterior sucker (0.047–0.05 mm. in diameter) equal to, or slightly larger than, ventral sucker. A stylet (Fig. 3) is present in the dorsal lip of the oral sucker, oriented at approximately a 45°–60° angle from longitudinal axis of the body. Stylet quite constant in size within each of the two populations, 0.023–0.024 mm. in cercariae from Coonamessett River and 0.021–0.0235 mm. in cercariae from Quashnet River. Stylet with lateral projections curved slightly upwards, about one-fourth the length of stylet from anterior end. Ventral sucker (0.042–0.048 mm. in diameter) located at about middle of the body, pedunculate, external margin bearing numerous serrate papillae. Prepharynx short; pharynx globular, 0.019–0.020 mm. in diameter, located mediad or slightly anteriad to the eyespots. Esophagus two to three times as long as the pharynx, bifurcation of the intestine just anterior to the midlength of the body. Caeca incompletely developed, usually reaching latero-posteriorly only to anterior edge of ventral sucker. Nervous system composed of a transverse band at the level of the pharynx with fibers extending to the eyespots. Eyespots well developed, brownish black. Three pairs of non-lobed penetration glands lie lateral and anterior to the ventral sucker. Each of the anterior pair of glands is drained by a duct which runs anteriad between the eyespots, while each of the two posterior pairs of glands has a duct which extends side by side anteriad between the body and the eyespots. Posterior and somewhat dorsal to the ventral sucker are the primordia of the genital organs abutting against the anterior end of the excretory bladder. Cystogenous glands appear to be absent and since no cyst is formed in caddis fly larvae, this might be expected.

The sac-shaped excretory bladder extends anteriorly almost to the ventral sucker. The wall of the bladder is thick, composed of numerous cells. Anteriorly two excretory canals enter laterally, proceeding along a sinuous path, from a point about mid-level to the ventral sucker where the posterior and anterior excretory ducts join. Ascending and descending ducts each drain three groups of flame cells: each group composed of four flame cells. The flame cell pattern is thus $2[(4+4+4)+(4+4+4)]$.

The tail is attached slightly ventrally and is variable in length. Usually it is a little longer than the body but it can be extended to over twice the length of the latter. When fully contracted, it is shorter than the body but it is never as wide as the body, thus differing from the cercaria of *A. isoporum* as described by Looss (1894) and by Dollfus (1949).

Measurements of the tails of several cercaria killed by pipetting them into hot formalin solution ranged from 0.20–0.34 mm. in length and 0.036–0.042 mm. in width. Both Looss and Dollfus pointed out that the tail of the cercaria of *A. isoporum* possessed an inner medullary portion, containing the nuclei, and a clear outer transparent cortical zone. The tail of the the cercaria of *A. alloneotenicum* also has a medullary and a cortical layer very similar to *A. isoporum*.

Measurements of both the body and the tail of the cercariae are extremely variable in living as well as in preserved specimens. No method of killing and fixing the cercaria was found which gave consistent results, so that morphological features such as sucker size, stylet shape and size, and size and extent of the excretory bladder are more reliable descriptive characteristics.

A single precocious cercaria which was 0.81 × 0.30 mm., with a tail 0.17 mm. long, was present in a redia 0.968 mm. long. Immature gonads were clearly defined. The stylet was absent in this precocious cercaria. Since the stylet was observed to aid in the escape of normal cercariae from rediae, this might account for the retention of the cercaria. The increase in size and development of gonads while still in the clam host is surprising.

Juvenile worms:

No cysts are formed although occasionally young worms became isolated in the gills of the caddis fly larvae and thus appeared cyst-like. Such worms seem to be prevented from reaching the haemocoel by the accumulation of detached tracheal vessels in the proximal part of the gill. Isolated worms typically cause the deposition of dark brown pigment by the larvae, which is a common response to any mechanical injury at any place in their bodies.

Experimental infections of caddis fly larvae isolated with individual clams liberating *A. alloneotenicum* cercariae resulted in the presence of numerous juvenile worms. One hundred caddis fly larvae were brought into the laboratory from sources thought to be free of infection; 50 were dissected and found to be negative, 25 were used in infection experiments and the remaining 25 were kept as controls and found to be negative upon dissection at the conclusion of the experiment.

In infections up to a week old, the worms varied from 0.27–0.33 × 0.13–0.17 mm. The pharynx was 0.02–0.021 mm. in diameter; the oral sucker 0.057–0.058 mm. and the ventral sucker 0.047–0.050 mm. In the larger specimens the genital primordium had begun to differentiate into identifiable reproductive organs. In later stages the testes had developed more rapidly than the ovary, similar to the development of these structures in other trematodes.

Eggs are present in infections 24 days old, but are few in number for an additional 14 days during which the worms continue to increase in size. When eggs are first produced, the worms are usually 1.5 × 0.62 mm. in size but the number of worms present in the larvae influences this size as well as the ultimate sizes.

DISCUSSION

Peters (1957) reviewed the genus *Allocreadium* and emended the generic diagnosis, retaining 16 of the 31 species described in the genus. He further listed 5 as *species dubiae* and transferred the remaining 10 species to other genera or left them as *species inquirendae* because of inadequate descriptions.

A. alloneotenicum conforms to the genus *Allocreadium* as emended by Peters (1957). It can be separated from the other species in the genus, however, by the extreme anterior position of the ventral sucker (within the anterior one-sixth of the body), and in the position of the testes (within the first half of the body). It specifically differs from *A. ictaluri* Pearse 1924, and *A. pseudotritoni* Rankin 1937 in lacking vitellaria in the forebody; by ventral sucker being larger than the oral sucker it differs from *A. handiai* Pande 1937, *A. nicolli* Pande 1938a, *A. kosia* Pande 1938a, and *A. mahaseri* Pande 1938b. *A. alloneotenicum* also differs from *A. transversale* (Rudolphi 1802) Szidat 1939, *A. schizothorcis* Pande 1938b, *A. lobatum* Wallin 1909, and *A. hasu* Ozaki 1926 in having the ventral sucker well within the anterior fourth of the body, and in the shape and position of the gonads. It differs from the type species *A. isoporum* (Looss 1894), and from *A. nemachilus* Kaw 1950 and *A. thapari* Gupta 1950 in the size and distribution of the vitelline follicles, the position of the cirrus sac, extent of the uterus and number and size of eggs.

The original descriptions of *A. markewitchi* Koval 1949 and *A. dogieli* Koval 1950 were not available for comparison but from the description of these species in Markevich (1952), it is apparent that *A. alloneotenicum* is distinct from these species.

A. alloneotenicum corresponds most closely to *A. neotenicum* Peters 1957. It differs in the shape of the body (always at least twice as long as wide), the posterior extent of the vitellaria and caeca, in the extent of the excretory bladder (which only reaches mid-level of the posterior testis instead of to under the anterior testis), and in the relative position of the ovary complex and the cirrus sac. *A. neotenicum* and *A. alloneotenicum* are unique in that they are the only two species within the genus known which apparently develop to sexual maturity in insects.

The clam, *P. abditum,* from Coonamessett also contained an infection of *Crepidostomum* sp. The cercariae from this infection were also ophthalmoxiphidiocercariae developing from rediae. They encysted both in nature and experimentally in the amphipod, *Gammarus* sp. *Crepidostomum* infections could be differentiated in the redial stages by the larger number of cercariae (usually approximately 36 being present) as well as by morphological differences.

The *Crepidostomum* cercariae possess a slightly smaller stylet, 0.017–0.019 mm., which has more of a median keel and slightly different lateral projections. They also possess 44–48 clearly defined cystogenous glands, 12–14 anterior and 32–34 posterior to the ventral sucker. The three pairs of penetration glands tend to be lobed. The excretory bladder does not extend as far anteriorly and the genital primordium is more extensive.

No fish were collected from this pool during the period November, 1956 to April, 1957. Attempts to infect various fish, including *Eucalia inconstans, Fundulus heteroclitus,* and *Salvelinus fontinalis,* with infected amphipods yielded only a limited number of juvenile *Crepidostomum* from the trout.

Infection experiments using the same three species of fish, feeding them caddis fly larvae known to be infected with juvenile *A. alloneotenicum,* were all negative. The worms were digested with the caddis fly larvae and portions of both could be recovered on the second day from the posterior portion of the gut of the fish. It might be possible that eggs would remain viable after passage through the intestine of a fish, but this was not investigated.

It appears, from the large number of eggs produced by the adult worms (up to 1200 eggs being recovered from a larva containing a single worm), that the infection in caddis fly larvae is the normal one for this species of *Allocreadium.* Infected larvae are never as active as uninfected ones; they are usually smaller and their cases show signs of neglect. The presence of numerous eggs throughout the body of the caddis fly larvae, including the appendages and the head capsule, plus the erosion and decrease in numbers and size of the fat-bodies make it extremely unlikely that an infected larva is ever able to pupate and reach adulthood. The recovery of the remains of dead larvae, still within the cases, enclosing empty egg shells of *A. alloneotenicum,* substantiates this view.

Peters (1955, 1957) stated that *Allocreadium neotenicum* from aquatic beetles from Michigan (possibly identical with species found by Crawford (1940) in aquatic beetles from Colorado) was a progenetic form that did not require a vertebrate host in order to complete its life cycle. He presented ecological evidence which supported his supposition. Buttner (1950, 1955) reviewed the progenetic trematodes and proposed four degrees of development of this characteristic. Peters (1957) added *A. neotenicum* to the fourth group of Buttner, that is, to the group in which the development of the gonads, the genital activity and the fecundity rivals that of the true adult. The example of this group cited by Buttner was *Paralepoderma brumpti,* a plagiorchid. *A. alloneotenicum* should be added to this group also. Both of these species of *Allocreadium* differ from the example cited by Buttner, however, since they develop to maturity in invertebrate rather than in vertebrate hosts.

The presence of ophthalmoxiphidiocercariae developing from rediae in sphaeriid clams in *A. alloneotenicum* supports the systematic scheme proposed by Dollfus (1949). Seitner's work (1951) should be re-investigated in the light of the results of the present study before final acceptance of the scheme of Dollfus. The controlled experiments on the life-history of *A. alloneotenicum,* supporting the morphological and ecological data presented by Looss (1894) and Dollfus (1949) for *A. isoporum* and by Peters (1957) for *A. neotenicum,* indicate a close relationship of the genera *Allocreadium, Crepidostomum,* and *Megalonia.* They all have ophthalmoxiphidiocercariae developing in rediae from sphaeriid bivalves thus forming a natural group.

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SUMMARY

Allocreadium alloneotenicum sp. nov. is described from the haemocoel of *Limnéphilus* sp., caddis fly larvae, from Cape Cod, Massachusetts. The life-cycle is demonstrated both in natural and experimental infections. The normal clam host is *Pisidium abditum.* Miracidia hatch in the debris from dead larvae or after ingestion and passage in the feces of the snail, *Aplexa hypnorum.* The process of miracidial peneration was observed. Secretions from the apical gland are histolytic in action, facilitating penetration, while the "penetration" glands produce the cuticula of the sporocyst. Sporocysts give rise to one generation of mother rediae which in turn liberate daughter rediae. Daughter rediae give rise to ophthalmoxiphidiocercariae and also produce occasional rediae. The cercariae penetrate caddis fly larvae (as many as 25 of them being found in natural infections). They mature in the haemocoel and a single worm was found to have laid 1200 eggs. Experimental infections of fish with infected caddis fly larvae were negative.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1957

ABSTRACTS OF SEMINAR PAPERS

July 2, 1957

Differentiation of cortical cytoplasm and extra-cellular membranes of oocytes, including changes at fertilization. Norman E. Kemp.

The cortical cytoplasm of the growing oocyte not only functions in selecting and transporting raw materials for new protoplasm and stored inclusions but also cooperates with surrounding follicle cells in the synthesis of the extra-cellular membranes, the vitelline membrane and chorion. It is not known whether the cortical cytoplasm of the oocyte actually synthesizes the materials for the vitelline membrane or merely serves as a form on which materials of follicular origin are deposited. Electron micrographs of developing oocytes of *Rana pipiens* and *Fundulus heteroclitus* have revealed fine details of the intimate morphological relationships between inwardly directed protoplasmic processes of follicular epithelial cells and outwardly directed protoplasmic processes of the oocyte. In the frog a layer of microvilli greatly increases the surface area of the oocyte, and some follicular processes extend into the layer of microvilli. Follicular processes within the microvillous layer may possibly connect with the surface of the oocyte, but connections have not been found at stages after oocyte and follicle cells are well separated. In the fish, protoplasmic processes extend from the oocyte through the zona radiata, and these processes either adjoin or are continuous with follicular cell processes within the subfollicular space. The student of fertilization would like to know (1) what membranes or jelly layers surround the egg and how they develop, (2) how sperm cells get through these membranes, and (3) how the cortical cytoplasm of the egg reacts to sperm entrance.

Lytic and other activities of the individual spermatozoon during the early events of sperm entry (Hydroides, Saccoglossus, and several other invertebrates).[1] Laura Hunter Colwin and Arthur L. Colwin.

In a number of invertebrate species the early events of sperm entry fall into two phases. It is suggested that *during the first phase,* which lasts only a matter of seconds, the following events occur: (a) the spermatozoon arrives at or near the jelly or membrane (whatever serves as a barrier around the egg); (b) the spermatozoon undergoes the acrosome reaction (of Dan) and sends its acrosome filament to or into the egg proper; (c) the acrosome filament delivers a stimulus to the egg; (d) the egg begins to react (*i.e.,* the fertilization reaction is initiated). It is not known how the filament succeeds in spanning the barrier or in what way the filament delivers the stimulus to the egg. The possibility that an enzyme (Bowen) or some other substance is carried into the egg by the acrosome filament should be examined. *During the second phase,* which in a number of species requires several minutes for completion, the acrosome filament and its attached sperm head, acting as one unit, move through the barriers and pass into the egg. Pits and spaces which appear in the egg membranes of *Saccoglossus* and *Hydroides,* as seen in living material and electron micrographs of thin sections, are interpreted as areas of erosion caused by egg membrane lysin emanating from the spermatozoon. It is suggested that the individual spermatozoon uses its own lysin to enable its relatively large head to pass through the barrier membranes, which the acrosome filament has initially spanned during the first phase of sperm entry.

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[1] Supported in part by a grant (RG-4948) from the National Institutes of Health, U. S. Public Health Service.

JULY 9, 1957

Membrane potential changes and ion movements in frog muscle. WILLIAM K. STEPHENSON.

Frog sartorius muscles soaked in potassium-free saline for 11 to 18 hours lost ½ their original fiber potassium and increased *ca.* 5 times their fiber sodium concentrations. Upon transfer to 10 mM KCl saline the fibers reaccumulated potassium and extruded sodium in equi-equivalent amounts of 31 mEq/liter fiber water within 50 minutes (recovery period). The mean electrical membrane potential remained constant at 40 mV during the recovery period.

Sodium extrusion is not directly responsible for the observed membrane potential since, in individual muscles, neither membrane potentials nor membrane potential changes were correlated with the amounts of sodium extruded. Two lines of evidence suggest that the observed potentials are not potassium diffusion potentials: (1) there was no correlation between potential changes and potassium accumulated during recovery, and (2) log fiber potassium plotted against membrane potential does not give the straight line relationship predicted by the Nernst equation. Results from individual muscles also indicate that the accumulation of potassium during recovery is an active process since: (1) the membrane potential during recovery was not large enough to cause the net passive influx of potassium, and (2) membrane potentials were not positively correlated with log fiber potassium.

Coupling of membrane potential to contraction in muscle. G. HOYLE.

The current tendency is to regard contraction as coupled to the membrane potential. In crustacean muscle this hypothesis can be tested probably more satisfactorily than in any other kind. There are two or more motor nerve fibers which effect different rates of contraction in the same muscle and there are also inhibitory fibers which can uncouple the excitatory action at the muscle.

The problem has been studied in conjunction with C. A. G. Wiersma. Muscles of eight species of decapod Crustacea were studied with the aid of intracellular recording. The nerve fibers were isolated and stimulated separately.

In many of the muscles, tension was associated with summating junction potentials which achieved a plateau of depolarization. The tension was closely related to the height of the plateau. Inhibitory nerve stimulation caused the level of the plateau to fall, the membrane potential returning towards the resting level. These observations therefore support the hypothesis.

But in muscles in the "paradox" state a contraction occurred at a low frequency of "slow" fiber stimulation. The largest responses were less than 0.5 mV and they were too widely spaced to summate. It did not seem possible that the depolarization was adequate to be the activating agent. This was supported by the fact that in the same muscle fiber the "fast" nerve fiber did not evoke a contraction at the same frequency of stimulation although it gave rise to large (12 mV) depolarizations.

Thus the contraction is coupled to some membrane "occurrence" which can be evoked (by "slow" fiber stimulation) or abolished (by inhibitory stimulation) more or less directly. The "occurrence" cannot be equated to membrane potential change. However, an adequate lowering of membrane potential has a similar effect.

Evidence for electrical inexcitability of neuron soma. W. H. FREYGANG, JR.

Grundfest has suggested that the portion of the neuron soma which is excited by synaptic transmitter substances does not produce a spike in response to an electrical depolarization. The dendrites and most of the somatic membrane of neurones in the mammalian central nervous system, as well as the cat's anterior horn cell, are covered with synaptic endings and, therefore, may not be electrically excited. If the intracellularly recorded action potential caused by antidromic excitation is produced by the spike of the axonal initial segment followed by the spike of a small area of electrically excited membrane on the soma, the impedance of most of the soma-dendritic membrane will not be altered. Since the resistance and capacity, as well as the transient voltage change across the soma-dendritic membrane, are known, the membrane current can be calculated. The calculated membrane current has a time course that is very similar to the externally recorded current, as measured from an external micropipette with its tip practically in contact with the soma-dendritic membrane. This finding supports the hypothesis that most of the soma-dendritic membrane is not electrically excited.

July 16, 1957

Further studies of the antimitotic and carcinostatic action of ovarian extracts. T. R. Tosteson, S. A. Ferguson and L. V. Heilbrunn.

The earlier work of Heilbrunn and Wilson showed clearly that saline extracts of the ovaries both of vertebrate and invertebrate animals could prevent mitosis in eggs of the worm Chaetopterus by keeping the protoplasm fluid and preventing the mitotic gelation. We then tried to apply this knowledge in the hope of finding a new type of carcinostatic agent. However, our early experiments on mice inoculated with Ehrlich ascites tumors were only occasionally successful and various attempts were made to purify the extracts so as to improve their efficacy. Finally after many unsuccessful experiments, we were able by fractional alcoholic precipitation in the cold to obtain preparations which markedly increased the survival time of mice inoculated with a highly lethal ascites tumor. The control of untreated mice, when properly inoculated, all died within 30 days. In experiments on thousands of mice we regularly were able to obtain a 30-day survival of about 20% of the mice injected intraperitoneally with the extracts. A 30-day survival is essentially equivalent to indefinite survival, for the mice that survive for this length of time were found on autopsy to be free from tumors. The extracts obtained by alcoholic precipitation are still highly impure. We are now able further to purify the crude extracts, and in some of our latest experiments 30–40% survival was obtained. The potent fractions derived from the crude extracts contain protein, lipid and carbohydrate. They are almost wholly insoluble in sea water, and probably for this reason their antimitotic action on Chaetopterus eggs is slight. However, even in very dilute solution they do tend to prevent the mitotic gelation.

The action of insulin on living cells. L. V. Heilbrunn, Francis T. Ashton, Carl Feldherr and Walter L. Wilson.

As yet, in spite of many attempts to show some effect of insulin on the enzymes extracted from living cells, no great success has been obtained. It is possible, therefore, that the primary action of insulin is on protoplasm and that the changes in the protoplasm affect the enzymic activity. This is rendered all the more probable by the many-sided evidence that colloidal changes in the protoplasm do markedly influence enzymic activity. This evidence is reviewed in a recent book, The Dynamics of Living Protoplasm (Academic Press, 1956). In ameba, sol-gel changes are constantly occurring, and this organism is therefore favorable for study. We used the giant ameba *Chaos chaos.* The surface precipitation reaction in ameba was found to be prevented by very dilute solutions of heparin. However if a dilute solution of insulin is added to the heparin solutions, the surface precipitation reaction is not inhibited. Using a solution of relatively zinc-free insulin, kindly supplied through the courtesy of G. H. A. Clowes by the Eli Lilly Company, we were able to demonstrate a very definite antagonism between insulin and heparin. Such an antagonism is further indicated by the fact that solutions of insulin prevent the metachromatic reaction of heparin with toluidine blue. Moreover although normally the amebae give a strong metachromatic reaction with toluidine blue, after they have been immersed for some time in solutions of insulin they no longer give this reaction. If, as we believe, the heparin or heparin-like substances of protoplasm act as a brake on the enzymic reactions of the cell, it is easy to understand why insulin would promote these reactions. In many experiments, we have also attempted to show an effect of insulin on the permeability of marine egg cells to glucose. As yet we have not been able to demonstrate any significant effect.

Magnetic studies on cells and protoplasm. F. T. ASHTON.

Magnetic forces can be used to study some of the properties of cells and protoplasm. Blood cells can be moved by strong magnets. When a dilute suspension of human blood cells in a small Petri dish was placed between the polepieces of a magnet with a field strength of 5202 gauss, within 12 hours most of the erythrocytes had migrated to a point between the polepieces.

Giant amebae (*Chaos chaos*) if fed paramecia with ingested iron, after digesting the protoplasm of the paramecia, will have vacuoles containing iron. If these are in the interior protoplasm, but not in the cortex, they can be moved by a magnet which gives a force of 980 dynes at a distance of one centimeter. Measurement of the force was accomplished by determining the distance at which the weight of small particles of iron on the pan of a microbalance was doubled. Using Stokes' law with necessary corrections, it was possible to measure the viscosity of the flowing protoplasm, although not as yet with any precision. A value of approximately 11 centipoises was obtained for the flowing protoplasm as a whole, or approximately 3 centipoises for the granule-free protoplasm. The interior protoplasm of the ameba gives no evidence of elasticity.

Attempts were made to shoot iron particles into the interior of sea urchin eggs by placing a magnet under a centrifuged mass of the eggs. Usually the eggs are smashed by this procedure, but in one case, an egg was made to contain a tiny iron rodlet. This could readily be twisted by a magnetic field, almost as readily as the magnetic rodlets outside the egg. On removing the magnet, both the rodlets inside and outside of the egg pointed to the magnetic north like tiny compasses.

The metachromatic reaction in various types of protoplasm. CARL FELDHERR.

At the present time, it is commonly believed that heparin is found only in mast cells. This opinion is due largely to earlier work which has shown that when sections of liver or lung are stained with toluidine blue, only the mast cells show a metachromatic reaction such as is given by heparin.

However it can readily be shown that many types of protoplasm give a metachromatic reaction with toluidine blue. Thus a very strong reaction is given by the cortex of the giant ameba, *Chaos chaos*. Likewise isolated nerve fibers of the lobster give a violet color when stained with dilute solutions of toluidine blue. Other types of cells which ordinarily show no metachromatic reaction may contain heparin or other metachromatic substances combined with substances which inhibit the metachromatic reaction. Thus frog muscle fibers stain blue with toluidine blue, but if they are previously treated with alcohol, if they are heated, if they are exposed to distilled water or if they are aged, they can be seen to give a metachromatic reaction. Similarly, the protoplasm of sea urchin eggs, ordinarily not metachromatic, becomes strongly metachromatic when the eggs are placed in distilled water. Nematocysts of hydra after treatment with alcohol also give a metachromatic reaction.

Electrophoretic mobility studies on irradiated fibrinogen. PETER RIESER.

Previous studies demonstrated that irradiation of purified bovine fibrinogen with 500 r causes a clotting delay upon the addition of thrombin. A reduction of 30% in the liberation of fibrinopeptide also occurs. Thus, a reduction in the generation of charged sites can be expected to occur. In order to determine whether a charge loss has occurred, an electrophoretic mobility curve of the irradiated and unirradiated protein was obtained. The conditions of electrophoresis were: protein concentration 12–15 g./l., dialysis against 0.1 ionic strength buffers containing 3.33 M urea. The mobility of fibrinogen irradiated with 500 r was decreased both above and below the isoelectric point. Since it is not likely that these results are due to an increase in the frictional ratio, the change in mobility is probably a consequence of a decrease in net charge. The difference in mobility may be converted into differences in net charge. A plot of Δh against pH shows that the curve levels off at 2.5, meaning that irradiated fibrinogen has lost that many negative charges. No leveling off appears to occur on the acidic side, but the charge difference cannot be much larger than 10 because of the relatively small shift in the isoelectric point. The latter is shifted in the acidic direction because the loss of cationic groups exceeds the loss of acidic groups. The cationic groups lost are probably imidazoles which have been shown by Mihalyi to take part in the polymerization.

Mechanisms of sol-gel transformations in the cytoplasm.[1] PAUL R. GROSS, SYLVAN NASS AND WILLIAM PEARL.

Certain *in vitro* properties of a Ca^{++}-initiated nucleoprotein aggregation reaction, obtainable in breis of sea urchin eggs or perfused rat liver, resemble closely the biochemical characteristics of the sol-gel transformation occurring in the cytoplasm *in vivo*. Fractionation experiments with both the marine egg material and liver homogenates show that the material which aggregates upon addition of small amounts of Ca^{++} is a nucleoprotein particle originating in the heterogeneous "microsome" fraction. A second fraction, ordinarily found among the soluble macromolecules in differential centrifugation, also precipitates under the influence of Ca^{++}, but this reaction differs kinetically and with respect to mechanism from that involving the microsomal nucleoprotein particles. The soluble phase particle, also a nucleoprotein, precipitates instantaneously with Ca^{++}, even at levels of 0.001 M Ca^{++} and less, but the reaction shows a primary kinetic salt effect, ceasing altogether at total ionic strengths lower than those probably obtaining in the living cell. Electron microscopic evidence is presented which suggests that the sensitive microsomal particle is identical with the "dense RNA particles" described by Palade and others in the cytoplasm of many cell types. A discussion of mitotic spindle structure is given, with micrographs which show that in the mitotic apparatus of the sea urchin egg, fixed by conventional buffered OsO_4 or with cold 30% ethanol and studied in ultrathin sections, the "Palade particles" comprise a major structural component.

JULY 23, 1957

Uricase inactivation by urea.[2] AURIN M. CHASE.

The inhibiting effect of urea on the oxidation of uric acid catalyzed by uricase (Worthington's semipurified preparation) was studied spectrophotometrically ($\lambda = 300$ mμ) in 0.1 M, pH 9 glycine buffer at 26° C.

Uricase undergoes an immediate, completely reversible inactivation by urea, similar to that reported by Osborne and Chase for *Cypridina* luciferase (J. Cell. Comp. Physiol., 1954) and by Chase and Krotkov for yeast invertase (same journal, 1956). Whereas, however, luciferase is completely inactivated by 1.5 M urea and invertase by about 3 M, the inactivation of uricase requires much higher urea concentrations and varies considerably according to the experimental conditions. For example, quite different results are obtained when borate buffer is used than with phosphate or glycine buffer.

In 0.1 M glycine buffer of pH 9 at 26°, no significant inactivation of uricase by urea occurs at concentrations lower than 3 M. Activity becomes increasingly less, however, as the urea concentration is raised above this value, and it is abolished at a concentration of about 8 M.

In addition to the immediate, *reversible* inactivation of uricase, the enzyme also undergoes an *irreversible* loss of activity when exposed to urea concentrations greater than about 4 M. This irreversible process apparently involves a primary, relatively rapid reaction and a secondary, slow one. The former is very dependent upon the urea concentration, its rate increasing about one hundred-fold between 4.5 and 7.5 M concentrations of urea.

The irreversible loss of activity of uricase during exposure to urea greatly resembles the situation observed by Simpson and Kauzmann for the effect of urea on the change in optical rotation of ovalbumin solutions (J. Amer. Chem. Soc., 1953), and the underlying mechanisms may well be similar.

Clotting of blood: A study in the polymerization of proteins. L. LORAND.

When fibrinogen is clotted by thrombin in the presence of calcium ions and the fibrin-stabilizing factor (FSF) of plasma, a clot is obtained which resembles the "plasma clot" in every respect. It cannot be dissolved in urea or monochloroacetic acid, in contrast to the soluble and mechanically weaker clot formed in the absence of the factor. Thus, the insoluble clot is regarded as a covalently cross-linked network, whereas the soluble kind is thought to be held together by secondary forces only (Lorand, 1954).

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[2] Aided in part by a grant from the National Science Foundation.

The fibrin-stabilizing factor has now been prepared from bovine plasma in a highly purified ($>$ 300-fold) form, as one of the globulin fractions giving virtually a single sedimenting boundary in the ultracentrifuge. The activity of the purified factor is protected by cysteine, but the latter cannot substitute for it. Fibrin-stabilizing factor contains titratable sulfhydryl groups, and all the evidence indicates that these may be essential for biological activity.

The insoluble clot, which in the case of the human and bovine species is probably the only naturally occuring one, was shown to be a co-polymer of fibrin and the fibrin-stabilizing factor in definite stoichiometric proportions. The co-polymerization of two different proteins, as illustrated by the above example, should be considered as a possible pattern for the biogenesis of other protein fibers.

This work was aided by a grant from the National Institutes of Health.

July 30, 1957

Effect of plant hormones on sea weeds. Luigi Provasoli.

Bacteria-free cultures of *Ulva lactuca* were obtained from small pieces of thallus treated with an antibiotic mixture. In a sea water medium enriched with vitamins and organic compounds, the zoospores formed filaments which never developed in the typical foliaceous thallus.

The filaments, after reaching various lengths, stop grownig, bleach almost completely, leaving a few intensely green dots, sparsely arranged. Upon transfer to a fresh medium, new filaments arise from these islands of pigmented cells.

Adenine, kinetin, indolacetic acid, and gibberellin, which affect morphogenesis in land plants, were then tried in the hope of obtaining the development of the typical thallus. This goal was only partially reached but preliminary experiments show a clear effect of the plant hormones on the germlings of *Ulva.*

Adenine, kinetin, and indolacetic acid favor the initiation of more filaments and affect the length of the new germlings. Adenine and indolacetic acid seem antagonistic at certain concentrations. Gibberellin dramatically promotes the elongation of filaments.

The response of a relatively simply organized sea weed to plant hormones promises that these morphogenetic determinants will be important factors for complex sea weeds. This response links even more tightly the green algae to the higher plants.

The variety of evolutionary steps towards increased morphological complexity in the algae offers the opportunity to choose appropriate organisms which, because of their relative morphological simplicity, may permit a better understanding of the mode of action of the plant hormones.

Sequence of changes in nucleic acids in synchronized cultures of Escherichia coli. Dwight McNair Scott.

Synchronization of division in cultures of *E. coli* in synthetic medium was achieved by chilling the cells at 6° for 45 minutes and returning them to 37°. The past history of the culture determined the possibility and time of synchronous division. Growing cultures, containing glucose, divided almost immediately after return to 37°. Growing cells, chilled in medium without glucose, divided about 60 minutes after glucose was added. Cultures which had exhausted glucose divided synchronously at 120 minutes, but only if they had been depleted at least six hours. During depletion, turbidity and DNA (by diphenylamine) remained constant, the count approximately doubled and RNA (by orcinol) decreased.

Methods of extraction of nucleic acids were investigated. Treatment of acid-washed cells with 5% perchloric acid at 4° for 17 hours extracted one half the RNA and left one half RNA and all the DNA in the residue (core). A variable proportion of DNA was made unreactive to diphenylamine by acid. Just before division the effect was slight but at midcycle approximately one half the whole cell DNA was affected. Acid treatment increased the color developed with orcinol (RNA), not exactly equivalent to the DNA decrease. These findings may indicate an unstable intermediate in DNA synthesis.

Frequent samples of synchronized cultures were analyzed to determine the time and extent of changes in nucleic acid components after glucose addition. Of interest are the following observations: 1) Increase at a logarithmic rate of bases as measured by absorption in the UV. 2) An immediate increase, up to 100% in 10 minutes in core RNA, then variations around a plateau and another rise before division. 3) A delayed increase in extractable RNA. 4) A variable time and percentage of rise in DNA. 5) Certain reciprocal relationships between core RNA and bases, core RNA and DNA, and core and extractable RNAs.

Some observations on the ribonuclease system in rat liver. Jay S. Roth.

The supernatant fraction of rat liver, obtained by centrifugation of a 1:10 homogenate of rat liver in water at 60,000 G for 90 minutes, contains a ribonuclease (RNase) inhibitor and an inactive RNase (i-RNase). These substances show the characteristics of proteins and have been purified and separated by the use of salt precipitation, calcium phosphate gel absorption and heat treatment. The RNase inhibitor is absorbed by calcium phosphate gel under optimum conditions; i-RNase is not, allowing 95–100% separation. The properties of the purified inhibitor and i-RNase have been investigated. RNase inhibitor is easily inactivated by heating, dilution with water, dilute acid and sulfhydryl reactants such as p-chloromercuribenzoate (CMB) and Pb^{++}. The inactivation by Pb^{++}, but not CMB, is reversible with hydrogen sulfide. Inactive RNase is stable to heating at pH 5 and 65° C. for 5 minutes, and is activated by this treatment as well as by treatment with hydrogen ion, pH 2 for 10–20 minutes at 25° C. Activation of i-RNase may also be accomplished by sulfhydryl reactants, the order of decreasing effectiveness being Pb^{++}, CMB, phenyl arsine oxide, iodoacetamide. The activation of i-RNase by sulfhydryl reactants is easily reversible with hydrogen sulfide. RNase inhibitor reacts competitively with yeast RNA and RNase. A new assay system has been developed which allows accurate simultaneous determination of i-RNase and RNase inhibitor. This is essential for purification studies where activation or inactivation of either component may occur during the purification process. The data suggest that RNase inhibitor is monovalent, that is, combines with one molecule of RNase. Thus, i-RNase is probably a complex of RNase inhibitor and alkaline RNase. (Aided by grants from the National Institutes of Health, Damon Runyon Memorial Fund and the American Cancer Society.)

August 6, 1957

Thymidine incorporation into the macronucleus of Euplotes.[1] Joseph G. Gall.

The hypotrich ciliate *Euplotes* has a single very large U-shaped macronucleus and a much smaller spherical micronucleus. Prior to macronuclear division a so-called reorganization band appears at the tip of each arm of the U and works its way slowly to the middle of the elongated nucleus. As seen in Feulgen-stained preparations these bands are composed of a lightly staining zone distal to a darker transverse line. In parts of the nucleus through which the reorganization band has passed, the Feulgen reaction shows an increased concentration of desoxyribose nucleic acid (DNA). To check the hypothesis that the reorganization band might reflect a progressive wave of DNA synthesis, rapidly dividing individuals were placed in a solution containing 5.5 μC/ml. of tritium-labeled thymidine. Thymidine is known to be a specific precursor of DNA and the tritium permits high autoradiographic resolution. After 8 hours growth in the label, the animals were squashed on glass microscope slides, stained by the Feulgen reaction, and covered with autoradiographic stripping film. After an appropriate exposure period the films were developed and made into permanent preparations. Radioactivity was found solely in those parts of the macronucleus through which a reorganization band had passed; thus there is a radioactive area distal to each reorganization band and a single non-radioactive zone between the approaching bands. The incorporation of thymidine into only those areas showing increased Feulgen staining suggests that the reorganization band represents the leading edge of a wave of DNA synthesis. Since reorganization bands are found in other Protozoa, the phenomenon described here is probably of general significance.

[1] Supported by funds from the National Science Foundation and the Graduate School of the University of Minnesota.

Interactions between chromosomes and cytoplasm during early embryonic development in Sciara (Diptera). C. W. METZ.

As indicated in earlier papers, Sciara exhibits an extraordinary series of phenomena which reflect apparently clear cut interactions between cytoplasm and chromosomes. One sequence involves specific chromosomal (genic) influences on the cytoplasm of the growing ovarian oocyte, such that later this cytoplasm acts selectively on particular individual chromosomes, inhibiting their mitotic activity and causing their elimination at specific times during cleavage stages in the fertilized egg. The work in question was done in our laboratory, largely by Anne Marie DuBois, M. Louise Schmuck (Mrs. Philip Armstrong), R. O. Berry and the writer. The basic genetic and cytological data are essentially complete. Since the problem is important and the material may be exceptionally favorable, present attention is focused on interpretations and on further extension of experiments to ascertain more about the underlying nature of the respective "influences."

In the fertilized egg of *Sciara coprophila* Lintner, the prospective soma includes all but the germinal "pole-plasm" at the posterior end. Somatic chromosome eliminations ordinarily occur in a peripheral clear zone of cytoplasm. The initial interpretation of the cause of these eliminations was based on Boveri's classical findings in Ascaris (pre-localization theory) by assuming a pre-localization of elimination-inducing materials in the clear zone in Sciara. However, more recent unpublished evidence of the writer indicates that elimination can occur normally in central areas of the egg and that all somatic eliminations may be under control of progressive physiological changes taking place uniformly throughout the cytoplasm. The distinction between the interpretations is important in relation to further work and our problem is to devise experiments which will eliminate one alternative or the other. The seminar report was designed to stimulate discussion of techniques and further procedures. Chromosome elimination in the germ line, by an entirely different process, was also considered.

AUGUST 13, 1957

Uptake of P^{32} in benthic algae in relation to primary productivity. EUGENE P. ODUM, EDWARD J. KUENZLER AND SISTER MARION XAVIER BLUNT, SNJM.

The rate of uptake of P^{32}, net productivity, respiration and gross productivity of large intertidal benthic algae were measured simultaneously in light and dark bottles suspended in a running sea water aquarium under controlled light and temperature. One microcurie of P^{32} and approximately one gram (dry weight) of alga were placed in each 500-ml. bottle of sea water under 450 ft. candles illumination at 21° C. for 3.5 hours. Uptake of P^{32} was measured by determining activity of the medium at intervals during the experiment, while productivity and respiration were determined from the initial and final oxygen concentration. Per cent uptake of P^{32} per hour per gram dry weight was similar in the light and dark for a given species, but markedly different between species. Rate of uptake was lowest (6–10%) in *Fucus vesiculosis* which also had the lowest gross production (0.8 ml. O_2 per gram per hour) and respiration rates and the lowest surface-to-volume ratio (approx. 22 cm^2 per cm^3). Uptake was highest in *Ceramium rubrum* and *Ulva lactuca* (32–52%) which had much larger surface-to-volume ratios and were four times as productive. Gross production/respiration (P/R) ratios were between 3 and 4 for all species under conditions of the experiment. It was evident that where environmental concentration of phosphorus is low, as is usual in sea water, tracer amounts of P^{32} are taken up at a rate characteristic for a given species and independently of light. Ability of a species to absorb the tracer appears to be related to structural features of the alga, high surface-to-volume ratio increasing the rate of uptake as well as increasing the ability of the plant to fix and utilize energy under favorable light conditions.

AUGUST 20, 1957

Biochemical studies of relaxation in glycerinated muscle. L. LORAND, J. MOLNAR AND C. MOOS.

Rabbit psoas fibers kept at 0° C. in 50% glycerol for two days and extracted further in 20% glycerol at 20° C. for 4–8 hours, develop maximal isometric tension on addition of 4 mM adenosinetriphosphate, and no spontaneous relaxation follows. Relaxation occurs, however, on adding 10 mM phosphoenolpyruvate (PEP) to the fibers, the diameter of which may be less than 80 μ ("single fibers"). Such relaxation is enhanced by K^+ similarly to the unique activation of pyruvate phosphokinase. Since the fibers contain this enzyme, PEP is believed to cause relaxation through transphosphorylating to adenosinediphosphate. But transphosphorylation alone is not sufficient, for fibers extracted in 20% glycerol for about 24 hours cannot be relaxed simply by adding PEP, in spite of the fact that the pyruvate phosphokinase is still present. Muscle contains an additional relaxation factor which is purified as follows. Minced rabbit muscle is homogenized in the cold with three volumes of KCl (0.15 *M*), and centrifuged at 1000 G. for 20 minutes. The supernatant is centrifuged at 1200 G. for 20 minutes to remove the mitochondria which have no relaxing activity. The "microsomes" are sedimented by a subsequent run at 41000 G. lasting one hour; the sediment is washed with KCl and finally suspended in KCl. On long extracted fibers, neither the transphosphorylase system nor "microsomes" give rise to relaxation, but jointly the two are very effective. The "microsomal" principle is thermolabile, is unaffected by ribonuclease, but is inactivated by trypsin, urea or desoxycholic acid. Since the latter does not affect the adenosinetriphosphatase of the particles, this enzyme cannot be identical with the relaxing factor. Apart from this, our findings are in agreement with those of Ebashi and Kumagai who studied isotonic relaxation with creatine phosphokinase and myokinase.

All relaxation studies to date (including the prevention of unloaded shortening of myofibrils by Portzehl) were carried out in the presence of a transphosphorylating system. The interaction of "microsomes" and the transphosphorylating system might give rise to a product which relaxes the fiber. As found for liver microsomes (Kenney, Colowick and Barbehenn), inorganic pyrophosphate, which is known to cause relaxation, might conceivably be produced under these conditions.

This work was aided by a grant from the Muscular Dystrophy Associations of America, Inc.

The dependence of creatine phosphate and adenosine triphosphate breakdown on work in iodoacetate poisoned muscles. FRANCIS D. CARLSON AND ALVIN SIGER.

Net creatine phosphate (CrP) and adenosine triphosphate (ATP) breakdown were determined, using the analytical methods of Ennor, and Strehler and McElroy, respectively, in anaerobic, iodoacetate-poisoned frog sartorius muscle at 0° C. (no rigor) as a function of the work done in a series of isotonic twitches under a 5-gm. load. Creatine phosphate breakdown varied linearly with work and no net adenosine triphosphate breakdown occurred until the creatine phosphate concentration dropped by 60% or more. Under a 5-gm. load .350 micromole of creatine phosphate was split for each millicalorie of work done, and the work done in a single maximal isotonic twitch was .821 millicalories per gm. of muscle. These data give a value of .287 micromole of creatine phosphate split per gram of muscle in a single isotonic twitch. On the basis of the heat studies of Hill it is possible to estimate the total energy output (heat plus work) for a single maximal isotonic twitch under a 5-gm. load. The value obtained is 3.54 mcal./gm. Since the work done is degraded to heat during relaxation the total energy released in a twitch appears as heat and so an estimate of the heat of hydrolysis of creatine phosphate in muscle at 0° C., pH 7.1, can be obtained. A value of 12,300 cal./mole results. Using a myosin content of 12%, a molecular weight of 440,000 for myosin, and the figure of .287 micromole of creatine phosphate split per gram in a single twitch, it is calculated that 1.05 creatine phosphate molecules are split per myosin molecule in a single maximal isotonic twitch with a 5-gm. load.

GENERAL SCIENTIFIC MEETINGS

AUGUST 26–29, 1957

Abstracts in this section (including those of Lalor Fellowship Reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read by Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

The influence of the branchial nerve and of 5-hydroxytryptamine on the ciliary activity of Mytilus gill. EDWARD AIELLO.

When the gill of Mytilus is excised, ciliary activity on the lateral epithelium of the gill filaments declines gradually for about one hour, at most, and then stops. A similar result is obtained by cutting the branchial nerve *in situ,* just distal to its origin at the visceral ganglion. Ciliary activity on the gill of the opposite, uncut side continues unabated for many hours. Cilia made quiescent in this way can be stimulated to renewed activity by crushing a small section of the gill filament. This resumption of activity spreads for about one mm. in all directions and lasts about five minutes. A hot water extract of gill tissue also activates quiescent lateral cilia and accelerates the beating of cilia that are already active. Veratrine sulfate 10^{-6} M and 5-hydroxytryptamine 10^{-7} M have similar stimulating effects. Gill tissue extracts are presently being assayed for 5-HT. These experiments suggest that lateral ciliary activity might be dependent on the release of some activating substance by tonic discharge of the branchial nerve.

Supported in part by a Predoctoral Fellowship from the National Institutes of Health and a grant to Dr. T. Hayashi from the Muscular Dystrophy Associations of America.

Motility in developing teleost embryos.[1] PHILIP B. ARMSTRONG.

The development of aquatic lomotion in *Ameiurus nebulosus* closely parallels that described for Amblystoma by Coghill. The earliest contractions of the skeletal muscle occur in the anterior segments of the body which is on the dorsum of the yolk and produces passive movements of the tail as a whole. Finally all of the muscle segments participate, a wave of contraction passes down one side of the embryo, a coil is formed as the tip of the tail passes over the dorsum of the embryo. There is no set and continuous regularity of alternation of these early contractions. None of the above movements are propulsive. They are seen prior to hatching.

At the time of hatching, propulsive contractions appear abruptly in which a wave of contraction extending down one side of the animal is so quickly followed by a wave down the opposite side that a coil cannot form. A rapid succession of alternate contraction waves produces forward motion.

Fundulus heteroclitus and *Opsanus tau* show a similar development of motility with some differences. Ameiurus is much the most active embryo showing considerable spontaneous activity, Fundulus considerably less. Opsanus is relatively lethargic.

Patterns of response and neural organization of supramedullary neurons of puffer (blowfish), Spheroides maculatus. M. V. L. BENNETT, S. M. CRAIN AND H. GRUNDFEST.

The supramedullary neurons of puffer respond in unison to stimulation of spinal cord, cranial nerves, dorsal (but not ventral) roots. This is shown by simultaneous intracellular recordings from random pairs of cells (see abstract by Crain, Bennett and Grundfest, this issue). With threshold stimuli or with stronger stimulation that evokes complex responses of many spikes at variable intervals, all cells fire nearly in synchrony. Direct intracellular excitation or stimulation of a cell with closely applied extracellular electrodes does not generally activate other cells. Irregularly in some cells, however, a direct spike is followed by a small potential. When this potential occurs a spike also appears in each of the other cells. These results are inconsistent with spread of excitation ephaptically or through protoplasmic bridges, and suggest synaptic mechanisms.

Cord section immediately above or below the cell cluster does not abolish synchronization. Division of the cluster by section involving the dorsal half of the cord eliminates synchrony between the caudal and rostral halves, but not within each half. Although responsiveness to stimuli distal to the section is lowered, both halves respond to rostral and caudal stimulations.

Activity of the cluster is followed by efferent impulses in cranial nerves and dorsal (but not ventral) roots. By tests of threshold, conduction velocity, and collisional extinction the efferent fibers belong in the same group as the afferents exciting the cluster. Since indirect stimulation is never observed to excite an individual cell, either the efferent impulses are transmitted across a synapse, or antidromic invasion of the cells is impossible.

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[1] This investigation was supported in part by NIH grant B-643.

No obvious functional loss follows chronic removal of the cluster.

While functional and anatomical relations of the supramedullary cells remain unknown, the electrophysiological data demonstrate that they cannot be sensory, as presumed by anatomists.

A morphological color change controlled by molting hormone in Lepidoptera. DETLEF BÜCKMANN.

A morphological color change preceding metamorphosis has been investigated in *Cerura vinula*. The larvae are green. When they stop feeding to spin their cocoon, they turn dark red because a red pigment is formed in the epidermal cells. After this, red pigment is formed in the fat body and the gut, too. The processes of pupal molt in the epidermis begin only 5 days after color change. Ligaturing experiments show that also only at this time the molting hormone is distributed in sufficient amount to cause pupation. The pupal molt is completed only 10 days after color change.

The color change can be prohibited by a ligature. Only the part of the body anterior to the ligature will redden. Evidently a factor causing color change is formed in the thorax, its distribution being prevented by the ligature. Extracts of the molting hormone Ecdyson, kindly provided by Dr. Karlson, München, injected into green abdomina of ligatured larvae in large dose (3000 Calliphora units) caused pupation without color change. Smaller doses caused reddening of fat body only. Very small doses (50 units) caused reddening of the epidermis only. So the normal course of events may be brought about by slowly increasing hormone concentration.

Thus it has been shown that Ecdyson not only causes molting in the epidermis. In small concentration it has quite a different effect. It acts on some metabolic processes leading to the formation of red pigments in epidermis, fat body and gut. The nature of the pigments is being investigated. They are ommochromes. The way ommochromes are formed in insects is well known. They are a product of trytophane metabolism. Ommochrome production preceding pupation has been found in other species, too. Presumably it is a characteristic of changes in metabolism preparing metamorphosis. Recent experiments tend to show that juvenile hormone also acts on ommochrome production.

Further studies in experimental hypothermia. C. LLOYD CLAFF, FREDERICK N. SUDAK AND MARVIN H. CANTOR.

Twelve-hour fasted male white rats injected (intramuscular) with Thorazine (25 mg./kg.) responded, in a cold environment of 6.5–7.5° C., with a fall in body temperature of 5° C. and a rise of 91% in metabolic activity as measured by CO_2 production. Animals treated with 2,4 dichlorophenoxyacetic acid (200 mg./kg., subcutaneously) responded to cold with a drop in rectal temperature of 3.0° C. but only a 54% increase in CO_2 output. Rats treated with Thorazine and 2,4-D in combination responded under the same conditions with a decrease in rectal temperature of 7.0° C. while CO_2 production increased only 15%.

At room temperature, rats treated with Nembutal (30 mg./kg., intraperitoneal) showed a decrease in rectal temperature as well as a lower CO_2 output. However, the peak metabolism of these animals, brought about by placing them in a cold environment (6.5–7.5° C.), was 102% above basal and body temperature fell 2° C. The peak CO_2 production and rectal temperature response of animals injected with Thorazine and Nembutal in combination were the same as those receiving either injection separately. Animals treated with a combination of Nembutal (30 mg./kg.) and 2,4-D (200 mg./kg.) responded to cold with a 10° C. drop in body temperature and no change in metabolic activity.

Studies made in an isothermic environment of 30.0° C. showed that the action of Thorazine and Nembutal causes a slight decrease in CO_2 production and no significant change in rectal temperature. On the other hand, animals treated with 2,4-D increase their metabolism 33⅓% while the temperature response was the same as a control animal. It was noted that the metabolism of

2,4-D treated animals was highly sensitive to any change in ambient temperature for a period of seven hours after injection.

Tissue transplantation in Pecten irradians.[1] JOHN E. CUSHING.

This work is part of a study initiated on sipunculids (Triplett, Cushing and Durall, unpublished data) concerned with the responses of invertebrates to transplants. The purpose is to learn more as to whether or not invertebrates synthesize antibodies. *Pecten* was selected because, while little work has been done on transplantation in mollusks, Butcher (1930) grafted eyes to gonads in this genus. As he gave few details, efforts were made to confirm and extend his observations. Transplantations were performed by removing mantle strips of a few mm., splitting the integument of the female portion of the gonad with the tip of a syringe needle, and tucking the strip ends under the edges of the cut, leaving the center portion exposed. Half the grafts so made took after 48 hours, whereas none took if completely buried, placed in the male portion of gonad, or under the mantle. Explants died in 48 hours, but established autografts have retained eyes, tentacles and contractility for over a month. A comparative series of auto and homo grafts survived as follows: start A 31, H 31: two days A 17, H 14: three days A 15, H 12: five days A 12, H 11: eleven days A 10, H 7: fourteen days A 9, H 5. This small series does not permit conclusions to be drawn, but does show the potential value of *Pecten* for transplantation studies. Of further value is the fact that grafts can be placed so that they deteriorate in a few days, permitting second graft series to be made.

Uptake of a radiomercury labeled diuretic (chlormerodrin) by the nephridia of Phascolosoma gouldi. ROGER L. GREIF.

The injection of chlormerodrin labeled with Hg^{203} into the coelomic fluid of *Phascolosoma gouldi* results in radiomercury accumulation in the nephridia in much higher concentration than in other tissues. This accumulation occurs when animals are kept in running sea water at 22° C., but if the worms are cooled to 5° C. for 12 hours prior to injection and are maintained thereafter at the lower temperature, the concentration of radiomercury in the nephridia will remain low. Upon transfer of the injected cooled animals to the higher temperature, accumulation of Hg^{203} in the nephridia begins. Diffusion of chlormerodrin within the coelomic cavity at 5° C. is not limiting. Dimercaprol (BAL) appears to decrease the affinity of the nephridia for mercury at 22° C. The relation of these findings to mercurial diuresis will be discussed.

Electron microscope observations on the cytoplasm of sea urchin eggs.[2] PAUL R. GROSS, DELBERT E. PHILPOTT AND SYLVAN NASS.

Fertilized, unfertilized, centrifuged, uncentrifuged, "normal," and injured eggs of *Arbacia punctulata* have been examined in thin sections with the electron microscope. Of several fixatives employed, the best for general purposes was found to be 1% OsO_4 in a veronal-buffered isotonic balanced salt solution, pH 7.4. Some elements of the mitotic apparatus are more easily studied in cold alcohol-fixed specimens, however. Among the structures studied were: pigment vacuoles, yolk particles, cytoplasmic vesicles of varying diameter, oil globules, mitochondria, double membrane systems in the cytoplasm, nuclear and outer limiting membranes, cortical granules, and small, dense particles such as described by Palade in mammalian tissue cells. These last are of average diameter 170 Å, with variation between 140 and 200 Å. The mitochondria show double outer membranes and "cristae," with osmiophilic layers 60 Å thick and total width of 150 Å. Pigment and yolk granules are surrounded by 60 Å membranes, but the yolk particles were occasionally seen surrounded by a double membrane of 150 Å thickness with osmiophilic outer components of 60 Å. The limiting membrane of the cell is peripheral to the cortical granules and about 100 Å thick, while the nuclear membrane is composed of a pair of

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[2] Supported by grants from the American Cancer Society, the National Science Foundation, and the Graduate School of New York University.

87 Å dense layers separated by a distance of 100 Å, although this latter dimension varies greatly to produce annuli, seen in tangential sections. In the centrifuged eggs, the classical descriptions of particle distribution hold, except that mitochondria are always found trapped in the centripetal oil layer as well as in the layer immediately centripetal to the yolk.

The effect of ions on the response of smooth muscle to cooling. RITA GUTTMAN AND SAMUEL ROSS.

The anterior byssus retractor of *Mytilus edulis* possesses both phasic and tonic systems. The slow tonic responses to rapid cooling were investigated by simultaneously recording tension and resting potential changes after ruling out the nervous elements by soaking the muscle in 10^{-4} M Banthine. Similar results were obtained with *Phascolosoma* and *Thyone*.

In these muscles cooling is not an effective stimulus unless the tissue is treated with subthreshold concentrations of potassium. The quantitative relations between the amount of cooling and the amount of associated depolarization necessary for contraction at various concentrations of potentiating potassium were established. The results can be expressed in a family of curves (one curve for each potassium concentration). The plateaus of the curves for sea water and potassium-free sea water were beneath the depolarization value necessary for contraction so that it is clear that no amount of cooling with sea water alone or with potassium-free sea water would ever be effective.

The effects of high and low sodium and of high and low calcium were also investigated. When the muscle is treated with subthreshold amounts of potassium and rapidly cooled in various concentrations of sodium ion and calcium ion, respectively, the sodium and calcium have no effect whatsoever upon the response. Acetylcholine, in subthreshold amounts, has a potentiating effect but, unlike potassium and cooling, acts through the nervous apparatus.

These results suggest that this muscle will respond to cooling with tonic contraction whenever a critical threshold amount of depolarization is achieved. Cooling alone cannot trigger the contraction since it cannot bring about sufficient depolarization. Cooling can result in contraction, however, if used in conjunction with some other subthreshold depolarizing agent. It is concluded that cooling affects the contractile mechanism indirectly by first causing membrane breakdown and depolarization.

Schooling behavior in mud snails in Barnstable Harbor leading to the formation of massive aggregations at the completion of seasonal reproduction.[1] CHARLES E. JENNER.

During the present summer, as in the preceding one (Jenner, 1956), snails on an extensive sand-mud flat in Barnstable Harbor, Massachusetts, underwent a striking change in distribution pattern—from a dispersed distribution, in which the snails were present over extensive areas of the flat, to an aggregated distribution, with snails occurring in massive aggregations. In both years the change took place at the same stage of reproductive activity within the snail populations. The timing of the termination of reproductive activity can be followed with great precision by making adequate samples and recording for females the per cent of those containing a formed egg case and for males the per cent having a fully developed copulatory organ (Jenner, 1956). The change in distribution pattern occurred during the first half of the transition period between the active reproductive phase (females with egg cases, males with developed copulatory organs) and the post-reproductive phase (females without egg cases, males with resorbed copulatory organs). It would appear that this change from dispersed to aggregated distribution is a regular aspect of the seasonal activity of these snails and is related to the state of reproductive activity within the population.

No concerted effort has been made to follow the history of the aggregations once they have been established, but it is now clear that the snails within these groups display schooling behavior, snails in any one part of the aggregation often moving in mass in the same direction. Such occurrences, often involving thousands of snails, present a dramatic sight. While such factors as currents, presence of food, and other physical and chemical stimuli are of importance in orientation in these snails, their schooling behavior cannot be accounted for solely in these terms. Clearly, social factors are involved.

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Protoplasmic bridges between follicle cells and developing oocytes of Fundulus heteroclitus. NORMAN E. KEMP AND EMERSON HIBBARD.

Continued work with the electron microscope permits us to amend the report presented by Kemp and Allen at the General Scientific Meetings of the Marine Biological Laboratory in 1956. We can now assert that in *Fundulus heteroclitus* there are direct protoplasmic connections between oocyte and follicular cells. What we formerly called microvilli within the zona radiata and subfollicular space are in reality not microvilli which simply adjoin the branching processes of follicular epithelial cells. Instead, these processes within the zona radiata are regional parts of intercellular bridges which are continuous from oocyte to follicle cells. The bridges form when follicle cells first separate from the surface of the oocyte, and they lengthen with increasing thickness of the zona radiata and subfollicular space. In view of this clarification of the nature of the protoplasmic bridges, we must reconsider the concept (Kemp and Allen, 1956) that the zona radiata is a product of the oocyte and that the chorion internum is therefore anatomically a vitelline membrane. It is possible that the materials for the construction of the zona radiata come at first from the follicle cells as they pull away from the oocyte. The zona radiata appears to thicken by apposition of fibrous matrix internally, *i.e.,* on the side next to the oocyte. It may be, however, that the materials for the matrix are transported inward from the follicle cells, by way of the protoplasmic bridges, rather than outward from the cortex of the oocyte. If the follicle cells are the primary source of the proteins used in constructing the zona radiata, then the chorion internum is not anatomically a vitelline membrane.

An effect of calcium-deficient Ringer on intact frog muscle. R. P. KERNAN AND A. CSAPO.

When frog toe muscle is soaked for about 10–15 minutes in Ca-deficient Ringer solution (0.18 mM/l.) and is then stimulated (in a 60 c/s, longitudinal a.c. field, for ½ second), the tetanus tension does not fall immediately when the stimulus is withdrawn but continues for several seconds. It is of interest to determine whether prolonged relaxation is accompanied by prolonged membrane activity or is due to a delayed cessation of the active state. Isometric tension and electrical activity of the membrane were recorded simultaneously on a dual beam oscilloscope so that the duration and amplitude of tension and action potentials could be compared directly. During electrical recording the muscle was removed from the bath and was stimulated through contact electrodes, placed at one end of the preparation. The pick-up electrodes were placed about 4 mm. apart at the other end of the muscle and were connected via a preamplifier (gain × 60) to the oscilloscope. The conditions in normal and Ca-deficient muscles were compared. Action potential of 13–17 mV accompanied tension in the normal muscle during stimulation. The electrical activity ceased at the end of the stimulus before the tension declined. When the muscle was stimulated after 10 to 15 minutes of soaking in Ca-deficient Ringer, the cessation of stimulation was followed by prolonged relaxation and also by action potentials of 4–7 mV declining slowly with tension decrement. Prolonged relaxation can be explained, therefore, by continued membrane activity without assuming a genuine prolongation of the active state.

Potassium contracture in frog twitch muscles. R. P. KERNAN AND A. CSAPO.

Tension and its rate of development were measured in the sartorius and toe muscles after immersion in high potassium Ringer solution. Maximum tetanus tension, elicited by a longitudinal electric field (½ sec., 60 c/s, a.c.), was used as a standard of comparison. Barkan-Boyle-Locke fluid in which the K level was varied from 24 to 120 m.eq./l. (substituted for Na) was introduced suddenly into the muscle bath to produce contracture. When contracture was completed the modified Ringer was replaced by normal Ringer. The contractures produced showed two phases, a rapid and a slow one. The initial fast phase could be abolished by curare or by pretreatment with 12 mM/l. K indicating that this phase is associated with propagated response and end plate activity. The second phase, which was smaller than the first, was unaffected by this treatment but its amplitude was found to be directly proportional to the external [K]. This suggests that diffusion through the interspace is a limiting factor in the K contracture. This point is further supported by the fact that for a given [K], smaller muscles developed relatively greater tension. In order to eliminate the effect of diffusion, toe muscles were reduced to small fiber bundles (approximately 15 fibers) and these were immersed in 96 to 120 mM/l. K Ringer. Contracture tensions then became almost equal to that of a normal tetanus. In small fiber bundles, tension and its rate of rise were directly proportional to the log of the external [K], suggesting that tension in K contracture is a function of the degree of depolarization of the fiber membrane.

Adaptation to salinity and temperature in a euryhaline hydroid. Otto Kinne.

The brackish water hydroid *Cordylophora caspia* (Pallas) is able to endure salinities from fresh water to nearly pure sea water (salinity optimum 15‰–17‰) and temperatures from about 4 to 25° C. In extremely suboptimal salinities (*e.g.*, fresh water) and in extremely supraoptimal salinities (*e.g.*, 30‰) the form of the colonies and especially the form of the hydranths alter considerably. Colonies with *identical genotype* consist in fresh water mainly of stolons; hydrocauli are short and unramified. In 15‰ and in 30‰, hydrocauli are longer and ramified. The most important alterations take place in the *hydranths,* the site of exchanges of water and ions, of oxygen uptake and excretion, of propagation, growth, etc. In suboptimal salinity and in supraoptimal salinity the hydranths become shorter and bear fewer and shorter tentacles. As the breadth is greatest in fresh water and decreases with increase of salinity, the hydranths are in fresh water somewhat globular, surface-volume relation of hydranths is low and increases with salinity. Surface-volume relation depends also on temperature; it is higher at 20° C. than 10° C. There occur remarkable alterations at the cellular level: fresh water hydranths consist at 20° C. of about 22,000 cells; these are columnar (high and narrow) and have a large nucleus. Epithelium of fresh water hydranths seems to be physiologically highly active and less permeable; 30‰-hydranths, on the other hand, consist of only 4–5,000 cells which are squamous (flat and broad) and have a small nucleus. Epithelium of 30‰-hydranths seems to be physiologically less active and more permeable. All these alterations caused by salinity are stronger at 20° C. than at 10° C. Cell-number is greater and nuclei are larger at low temperature.

Inorganic pyrophosphatase activity of glycerinated muscle. C. Moos and L. Lorand.

In the course of investigating relaxation in glycerinated muscle fibers, a study was undertaken of the interaction of inorganic pyrophosphate (POP) with glycerinated muscle, and in particular, of the pyrophosphatase (POPase) activity of this material. Samples of glycerinated muscle, prepared by extraction in cold 50% aqueous glycerol as described by Szent-Györgyi, are homogenized in 0.1 M KCl containing 4 mM $MgCl_2$ and buffered at pH 7.0 with 10 mM imidazole. Upon addition of POP to the suspension, orthophosphate is liberated at roughly a constant rate until the POP substrate is nearly exhausted. This rate is, in rough order of magnitude, about 1% of the rate of hydrolysis of adenosine triphosphate (ATP) under similar conditions. It has been found that POPase activity is retained in glycerinated muscle stored at −20° C. for as long as 18 months. The activity is completely extracted from the muscle upon homogenization in the above buffered salt solution, but homogenization in water leaves a major fraction of the activity in the insoluble residue. The POPase activity of the homogenate is inhibited by low concentrations of calcium; using 1.0 mM POP, 0.2 mM $CaCl_2$ causes more than 60% inhibition, and 1 mM $CaCl_2$ inhibits over 90%. Salyrgan also inhibits POPase, but only partially; all concentrations of the drug above 0.2 mM reduce the activity by only about 70%, while ATP hydrolysis is more than 99% inhibited under these conditions. Utilizing these facts, the effect of added ATP on POPase activity was investigated, and it was found to have no effect. Adenylic acid, which could be studied without salyrgan, was also without effect.

This work was aided by a grant from the Muscular Dystrophy Associations of America, Inc.

Metabolic consequences of a genetic block between α-ketoglutarate and succinate in Escherichia coli. ARNOLD L. NAGLER, ELIZABETH S. MINGIOLI AND BERNARD D. DAVIS.

In a search for auxotrophic mutants of *Escherichia coli,* using ultraviolet irradiation and selection by means of penicillin, a new kind of mutant was obtained. This strain (309–1) grew under anaerobic but not under aerobic conditions when the mineral-glucose medium was supplemented with certain biosynthetic products of the 4-carbon dicarboxylic acids: lysine plus methionine or threonine. (A biosynthetic precursor of these compounds, aspartate, was inhibitory.) Succinate supported even more rapid growth and was also effective, alone or with glucose, under aerobic conditions. Glutamate was inactive. Furthermore, this mutant, growing on glucose plus succinate, heavily fed a strain that responds to only α-ketoglutarate or glutamate. These findings suggested that mutant 309–1 is blocked between α-ketoglutarate and succinate. This conclusion was established by showing in a Warburg respirometer that non-growing cell suspensions of mutant 309–1 failed to oxidize glutamate to CO_2, whereas the wild type carried out this conversion almost quantitatively. In contrast, succinate was oxidized at a similar rate by the two strains.

These results throw further light on the metabolic role of the tricarboxylic acid cycle in *E. coli.* Previous studies (Gilvarg and Davis, 1956) have shown that a mutant blocked between oxalacetate and citrate requires glutamate but not any direct biosynthetic products of the dicarboxylic acids. The present findings show that when these 4-carbon acids are being further metabolized to α-ketoglutarate without being regenerated from that compound, the rate of their formation from glucose is not sufficient to meet the biosynthetic needs of the organism.

Contractility of the hyaline layer of Arbacia punctulata. A. K. PARPART AND JULIEN CAGLE.

The hyaline layer of fertilized *Arbacia punctulata* eggs is probably a polysaccharide and is contained in a polymerized state in the cortical granules prior to fertilization. Fertilization or parthenogenic agents cause rapid and explosive depolymerization followed by slow polymerization, to form the hyaline layer, about 2 μ thick, closely surrounding the egg.

This hyaline layer exhibits remarkable contractile properties when the pH of the environment is decreased. At pH 4.0 slight contraction occurs. However, at pH 2.4 a rapid and strong contraction occurs. It shrinks down to a thin line tightly compressing the egg and is capable of pulling two- and four-cell stage blastomeres tightly together. Several acids added to sea water cause this. The force of contraction is so great that the plasma membrane and cytoplasm of the egg are forced through numerous minute apertures in the hyaline layer to form ca. 3 to 5 μ blebs over most of the egg surface. The contraction of the hyaline layer is reversed in sea water and this contraction and relaxation can be repeated a number of times.

The blebbing that follows active contraction is not due to the acidity of the environment. This was established by depolymerizing the hyaline layer in isosmotic sucrose and then exposing the egg to isosmotic sucrose brought to pH 2.4. This induced a mild degree of polymerization of the hyaline polysaccharide but no blebbing.

The hyaline layer is not stainable by methyl green, which suggests it is not a sulfate-polysaccharide. It is stained *in vivo* by methylene blue, toluidine blue, pyronin and neutral red, which suggests it may be an acid-polysaccharide. It is not stainable by phenol red or Janus green B. Methylene blue, added to depolymerized hyaline layer *in vivo,* causes a partial polymerization. This suggests the polysaccharide may be related to the lichenins.

Observations on the histology and oxidative metabolism of gill cartilage from Limulus polyphemus. PHILIP PERSON AND ALBERT FINE.

Limulus gill cartilage, a mesenchymally derived endoskeletal tissue, is histologically very similar to vertebrate hyaline cartilage. Gill cartilage growth and development involves differentiation, maturation, and degeneration of cells, such as are encountered in the life history of a typical vertebrate cartilage. It is of interest that there may be found, in this invertebrate tissue, sequences of cell development typically associated with vertebrate endochondral ossification, with the exception that *Limulus* cartilage is never replaced by a process of ossification. In homogenates and slices prepared from cartilage from larger animals, six inches in length or more, it was not possible to demonstrate direct oxygen uptake in the presence of substrates such as glucose, succinate, ascorbate, and hydroquinone, using as much as one gram, wet weight of tissue, per Warburg vessel. A wide variety of suspending media was used in air and oxygen atmospheres. Inability to demonstrate terminal oxidase activity by manometric and spectrophotometric methods was not considered unusual in view of the severe degenerative changes seen in the cells of cartilage taken from larger animals. In specimens approximately one and one half to three inches in length, the gill cartilages resemble early embryonic cartilage, histologically. Homogenates prepared from such tissue demonstrate aerobic utilization of succinate and hydroquinone. Malonate, equimolar with succinate, inhibited oxygen uptake 100%. Hydroquinone utilization was inhibited 56% by 10^{-4} *M* cyanide and 32% by 10^{-4} *M* azide (final concentrations). Spectrophotometrically, such homogenates could reduce cytochrome *c* in the presence of succinate and 10^{-3} *M* cyanide; and could oxidize reduced cytochrome *c*. This marks the first demonstration of succinoxidase and cytochrome oxidase activity in cartilage tissue from any source.

Studies on the distribution and properties of the ribonuclease system in marine forms. JAY S. ROTH AND DOROTHY BACHMURSKI.

Previous studies on the ribonuclease (RNase) system in rat liver have demonstrated the presence of two active enzymes, an enzyme inhibitor and an enzyme-inhibitor complex. The occurrence of different RNase systems in lower forms, correlated with other biochemical factors, may give some insight concerning the physiological purpose of these systems which occur, apparently, in most animal cells.

Various tissues were homogenized in ice-cold water and a supernatant fraction prepared (except with sperm) by centrifugation at 60,000 G for 30 minutes. The supernatant fraction was assayed for RNase activity. RNase inhibitor and inactive RNase (i-RNase, RNase-inhibitor complex) and the homogenate was assayed for RNase activity at pH 5.6, 6.4, 7.0 and 7.8, and after heating for 5 minutes at pH 5.6 and 7.8. All assays were by previously published methods using ABC buffer.

In a series of experiments on marine eggs and sperm, i-RNase but no RNase activity was found in the supernatant fractions. With starfish nucleoli (supplied by Dr. Walter Vincent) the specific activity of both RNase and i-RNase was the highest encountered, which is of considerable interest in view of the rapid RNA metabolism in this particulate. RNase inhibitor was detected, in small amounts, in *Chaetopterus* eggs or sperm, the greatest activity being measured at pH 7.0 or 7.8.

When the supernatant fractions from some higher forms were examined, all contained from moderate to large amounts of alkaline RNase activity in contrast to rat liver, which contains little. This RNase activity was strongly inhibited in squid gill and starfish gonads by 4×10^{-4} *M* p-chloromercuribenzoate indicating that the RNase was of a sulfhydryl nature as contrasted to mammalian enzymes which are not. No i-RNase was detected, but RNase inhibitor was widespread, and both acid and alkaline RNase activity were detected in most specimens. (Supported by grants from National Institutes of Health, American Cancer Society and Damon Runyon Memorial Fund.)

The effect of sugars on gastrulation of Chaetopterus embryos. DWIGHT B. MCNAIR SCOTT.

During the course of investigation in previous summers on the carbohydrate metabolism of the developing Chaetopterus embryo we attempted to measure the production of labelled CO_2 from glucose-1-C^{14} and found that the presence of glucose in the sea water had an adverse effect on the subsequent development of the larvae. Therefore the effects of different concentrations of glucose, fructose and sucrose, added at different times before and after fertilization, have been investigated. Fertilized Chaetopterus eggs hatch at about 7 hours and the trochophores invaginate at 33–36 hours. Glucose (0.0055 *M*), added before fertilization and at times up to 9 hours after fertilization, caused a slight delay in the time of gastrulation which otherwise was normal.

Glucose (0.014 *M*) delayed the onset of gastrulation, decreased the size and functions of the alimentary tract, decreased the proportion of embryos completing gastrulation and prevented later growth of the larvae in size. Glucose (0.028 *M*) added at any time up to 7 hours (hatching) prevented gastrulation; and addition at 9 hours and at 10 hours delayed gastrulation to the fourth day and the third day, respectively. These larvae did not increase in volume beyond that of the egg. Concentrations 0.045 *M* and above prevented any development beyond the trochophore and produced abnormal forms. Transfer of the embryos from glucose to sea water after three hours or more did not lead to normal gastrulation. Sucrose, 0.03 *M*, produced no effect on the time or course of larval development. Thus these results are not comparable to those reported by K. Dan and A. R. Moore with Dendraster or Arbacia eggs. Their sucrose concentrations were higher and produced abnormal invagination and exogastrulation by osmotic effects.

The effects of glucose are more comparable to the effects of amino acids on marine eggs as reported by Mathews, or H. D. King.

Studies on the interactions of the bound nucleotide of actin. RICHARD C. STROHMAN.[1]

An investigation was carried out on the changes in the bound nucleotide of actin associated both with actin-actin and actin-myosin interaction. The possibility that the bound nucleotide was also available for interaction with creatine phosphate (CP) was also studied.

It was found that a reversible depolymerization of actin can be obtained by dialysis against CP in the presence of creatine phosphokinase. During depolymerization the ADP of the F-actin was converted to ATP. If, however, F-actin is reacted with CP but under conditions where the actin remains polymerized then no such transphosphorylation takes place.

The possibility that the classical depolymerization of actin with ATP might also occur through a transphosphorylation, where ATP is the phosphate donor, was subsequently investigated. Depolymerization was run using C_{14} ATP and it was found that there was no incorporation of radioactivity into the bound nucleotide. Further studies are in progress on this system but the tentative conclusion is that the mechanism of nucleotide change is one involving transfer of the terminal phosphate of ATP to the ADP of the actin.

It is possible to show that G-actin forms a complex with H-meromyosin in which the ATP of the actin is converted to ADP. The nucleotide of the complex is still able to interact with the creatine-phosphokinase system since regeneration of ATP is observed when CP is added under the proper conditions. In F-actin-H-meromyosin complexes the nucleotide is unable to interact with CP under the conditions used. G-actin-H-meromyosin complexes are thus able to carry out a turnover of the terminal phosphate of the actin nucleotide in the presence of a phosphate donor. No such turnover can as yet be demonstrated for F-actin-H-mero-myosin complexes.

Electrical recording in the living squid. ROGER E. THIES.

The resting potential and spontaneous activity of the giant axon were measured in *Loligo pealii.* Animals were maintained for many hours strapped under the lid of a lucite box containing oxygenated sea water. The axon was observed near the rostral margin of the fin with transmitted light. To insure visibility the ink sac duct was ligated and the skin removed locally.

Spontaneous activity of two animals was recorded between two glass-insulated silver wires plunged into the mantle muscle just above the axon and one centimeter apart. Irritation by poking the head caused firing of the giant fiber. The resultant axon potentials of 0.2–0.4 millivolts were followed after 2–3 milliseconds by muscle potentials of 1 to 5 millivolts. The potentials occurred singly or in bursts of up to eight. The longer trains began at frequencies of 140 per second and declined to 50 per second, with the total duration never exceeding 100 milliseconds. During continuing irritation such bursts recurred at one- to five-second intervals.

Resting potentials were measured with the squid in a closed system of circulating sea water cooled to 8° C. The resulting decreased rate of mantle contraction minimized movement of the axon during penetration with KCl-filled micropipette electrodes. Removal of muscle from above the fiber allowed penetration of the axon membrane under direct observation. Values of 60 to 111 millivolts were measured in sixteen penetrations of six axons, with a mean value of 77 millivolts. Three axons gave values of 52 millivolts and below.

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[1] Supported by fellowship from U. S. Public Health Service to the author and by a grant to Dr. T. Hayashi from the Muscular Dystrophy Assn. of America.

The high resting potentials may be due to the elimination of the mechanical trauma associated with removal of the giant axon, as well as preservation of all the animal's functions. Prosser and Young showed that single impulses in the giant axon give maximal contraction. Yet apparently the squid frequently uses short bursts to insure effective contraction.

Glucuronidase and sulfatase of mollusks. WALTER TROLL.

Glucuronidase and sulfatase are useful reagents in the study of mammalian metabolism, since a number of compounds such as steroids and aromatic amines are excreted as glucuronides and sulfate esters in the urine. Recently a number of English workers have reported that mollusks occurring at their sea coast are excellent sources of these enzymes. We have confirmed this observation with several mollusks obtainable here. A good source appears to be the liver of Mactra homogenized with water. Both glucuronidase and sulfatase are readily purified by ammonium sulfate fractionation yielding enzyme preparations with 50-fold the activity of the most purified mammalian preparations. The optimum pH for glucuronidase using phenolphthalein glucuronide as the substrate is pH 4. The optimum pH for the sulfatase using p-nitrophenol sulfate as the substrate is 5.6. The sulfatase is inhibited by phosphate and sulfate ions. All these properties are identical with the ones reported by the English workers for a variety of molluskan enzymes. These observations can be interpreted to indicate that these enzymes are characteristic constituents of mollusks.

Proteins of starfish nucleoli. W. S. VINCENT.

Among the many unknown things about nucleoli is where the nucleolar materials come from. As about 95% of the nucleolar materials are protein, an analysis of this component might suggest some answers to this particular basic problem.

The major protein component of isolated starfish nucleoli proved to be insoluble in reagents which might yield solutions suitable for ultracentrifugal or electrophoretic analysis, so the technique of end group labelling with dinitrofluorobenzine was used. When nucleoli were reacted with this reagent, the hydrolysate, upon chromatographic separation, yielded only a single amino acid (as yet unidentified) labelled with the dinitrophenol. A control reaction on acetone powder of whole starfish eggs yielded some half-dozen labelled amino acids.

Although the possibility is not completely excluded that the free amino end groups of some of the protein species may not be available to the reagent, the simplest interpretation of these experiments is that the nucleolus consists of a single protein. If this simple explanation is true, then it is likely that the nucleolar protein is the product of a single genetic "site" rather than being the accumulated products of many different chromosomal regions.

Survival of marine invertebrate cells in tissue culture (Limulus and Ostrea). ANNE WARWICK AND F. B. BANG.

Limulus amoebocytes were cultured in varying dilutions of Limulus serum in roller tubes with 100 units of penicillin and 100 units of streptomycin at room temperature. As Loeb has shown, the amoebocytes maintain their oval, granular appearance in undiluted Limulus blood. In this medium a prompt loss of granules and change in shape of the cells were obtained with a bacterial toxin within 15 minutes. With renewals of medium, Limulus cells survived more than 30 days in 10% Limulus serum in artificial sea water. Similar observations were made in 25, 50 and 100% Limulus serum. In both living and Giemsa-stained preparations no mitotic figures were observed. In all of the cultures there was a gradual decrease in the mass of cell material. Attempts at transfer by explantation of recently formed clots, by trypsinization, in Ca-free sea water and Versene treatment failed. Oyster amoebocytes obtained by cardiac puncture survived in their own serum for 5 days. Mantle tissue was explanted in artificial sea water, 10% and 25% Limulus serum in artificial sea water with 10 mg% glucose, and in hemocyanin-free Limulus serum. Following the early migration of amoebocytes from the mantle tissue, in the sea water there was a degeneration of all of the cells by two days. The addition of the Limulus serum was accompanied by the organization of mantle epithelial cells, the formation of ciliated cysts within the mantle and ciliated borders. Such ciliated cysts remained spinning for 8 to 10 days and the amoebocytes persisted for 12 days. Again no mitotic figures were seen and the cultures gradually degenerated. Some muscle cells apparently also persisted since contraction of the explant was observed as late as 10 days.

Method of analysis of a "gene" in Mormoniella. P. W. Whiting.

A restricted region of the germ plasm, a gene so-called, may be studied by the interaction of mutant alleles in the compounds. The allelic series is likely to be complex, affecting more than one factor. The different mutant states of the factors constitute sub-series of alternatives within the series of pleiotropic allelic genes. These states cause impairment of function of greater or less severity. Alleles may be produced by irradiation of wild type and isolated by subsequent crossing to a stock with a recessive marker. Thus, irradiated wild-type Mormoniella males have been crossed to females with the *R*-locus eye color peach-333.5. A mutant-type daughter will be a compound of peach and a new *R*-locus mutant gene. Wild-type daughters indicate either no mutation, mutation at loci other than *R*, or *R*-locus mutation in factors other than those affected by the marker gene. Peach-333.5 is a triple recessive gene, mutant in eye-color factors *O, S* and *N* but not in *M*. Mahogany-846 is recessive in *M* alone. The compound female is, therefore, wild type—$o.s. + .n/+ . + .m. +$. There have also been found in this region at least three factors, *A, B* and *C*, affecting viability and sterility. Mutations in *A* may be female-sterile, *fsa*, or lethal, *la*. The compound, *fsa/la*, is a viable but sterile female like the homozygote, *fsa/fsa*. Some of the *R*-locus alleles suppress crossing-over with purple body, *pu*, eleven map units distant. It is postulated that a similar method of analysis applied to other regions also would produce pleiotropic alleles and convert these regions into loci by suppressing crossing-over within them and thus integrating their factors into segregating units, genes.

PAPERS READ BY TITLE

Energy metabolism and ciliary activity of Mytilus gill. Edward Aiello.

In 1924 James Gray reported that veratrine stimulated both ciliary activity and oxygen uptake of excised Mytilus gill to about 150% normal and suggested a direct effect of veratrine on energy metabolism. It has been found that veratrine sulfate 0.01% causes no significant change in the oxygen uptake or ciliary activity of gills whose cilia have been totally inhibited by 2,4-dinitrophenol 10^{-3} *M* or high salt (3 × normal osmolarity) or partially inhibited by KCN 0.01 *M*. It does, however, almost totally restore the oxygen uptake and ciliary activity after inhibition by sodium azide 0.01 *M*. Azide, in turn, only slightly inhibits the oxygen uptake of gills in DNP or high salt but greatly inhibits it, and ciliary activity, in natural sea water. The absolute values of oxygen uptake in DNP or high salt with azide present are higher than those with azide alone although ciliary activity is only present, but weak, in the latter. Veratrine does not restore ciliary activity that has been inhibited by lack of oxygen. The simplest explanation seems to be that veratrine effects oxygen uptake only indirectly through its effect on ciliary activity; also, that azide effects oxygen uptake directly only to a small degree and mainly by a direct inhibition of the ciliary mechanism. This latter action is the only one reversed by veratrine. A further implication is that, depending on circumstances, the degree of ciliary activity regulates oxygen uptake, *e.g.* with veratrine stimulation, or the rate of oxygen uptake determines the degree of ciliary activity, *e.g.* with cyanide inhibition.

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Reaction to injury in the oyster. F. B. Bang.

Amoebocytes of the oyster readily phagocytize a variety of marine bacteria *in vitro*. However, many strains of marine bacteria were not phagocytized under the same conditions. When phagocytosis took place it was preceded by clumping of bacteria on the amoebocytes and frequently by the sticking of the bacterial flagella on the processes of the amoebocytes (electron microscopy). The formation of an extracellular clot with oyster amoebocytes was demonstrated frequently in blood obtained from well-fed oysters which had been kept from feeding during the preceding 6 to 12 hours. This extracellular clot entrapped bacteria and spread from the amoebocytes throughout and around the cellular clot. This extracellular clot was also observed *in vivo.* Intravascular clotting (white cell clumping) was observed in the vessels of the living oyster following trauma directly to the oyster or in badly traumatized oysters opened on the half shell. It was produced regularly by the intracardiac injection of an extract of oyster tissue (gill). This intravascular clotting consisted of clumping of white cells, the sticking of these clumps to the walls of the vessels and the contraction and thrombosis of large vessels which lasted for one to two hours. Control injections of sea water and carmine failed to produce these effects. Heated extract and bacterial suspensions produced temporary effects (10 to 15 minutes).

Lethal irradiation of Tillina magna in its active and encysted states. JOSEPHINE BRIDGMAN.

The ciliate *Tillina magna,* in its active state, is somewhat more sensitive to x-irradiation than are many other protozoa for which lethal doses are known, but like the other protozoa tillinas vary considerably among themselves in susceptibility to radiation. This is particularly conspicuous if they are irradiated at different times in their life cycles.

Tillina, like the better known *Colpoda,* is very easily induced to form cysts. These are smaller than the active form and in them the animal is de-differentiated. In a series of tests active tillinas and tillina cysts, in groups of twenties, were irradiated at intensities of 175, 200 and 225 kr, doses chosen because they are on the borderline of survival for these animals. It is well known from earlier work on *Tillina* that animals which initially survive a dose of irradiation often go subsequently into an abnormal cyst from which there is no revival. The fate of the active animals irradiated at these doses was often such a cyst. The effect of irradiation on a normal cyst is not immediately apparent. However, unless such a cyst, under circumstances favorable to excystment, becomes active within twenty-four hours, it is unlikely that it is alive.

Examination, after twenty-four hours, of the animals treated as described above indicated in every case a higher percentage of survival in the animals irradiated as cysts than as active animals. In one typical experiment active animals irradiated at 225 kr showed 10% survival after 24 hours and cysts treated at the same time to the same dose had a 75% survival. Similar differences between susceptibilities of active and encysted forms were shown at the other doses. The experiments as a whole show clearly that active animals are more susceptible to x-irradiation than animals in resting cysts.

Work done with the support of the A. E. C. under contract No. AT–(40–1)–1818, with Agnes Scott College.

The inhibition of the cardiac ganglion of Limulus polyphemus by 5-hydroxytryptamine. A. S. V. BURGEN [1] AND S. W. KUFFLER.

The rhythmic bursts of activity in the isolated cardiac ganglia of *Limulus polyphemus* were slowed by 5-hydroxytryptamine (5-HT) 5×10^{-6} g./ml. and arrested by 2–3 times this concentration. The inhibitory effect of 5-HT could be prevented by roughly equal concentrations of bromlysergic diethylamide (BOL) which by itself had no striking effect on the activity of the ganglion. Gamma-aminobutyrate had a similar action to that of 5-HT but was about 100 times less active.

Larval development of Streblospio benedicti Webster. MILDRED A. CAMPBELL.

Early developmental stages of *Streblospio benedicti* occur within a protected area on the dorsal surface of the middle segments of the adult. In the living material these segments appear to form a brood chamber with dorsolateral folds of the body wall, but definite information must wait upon histological sections. The eggs are blue-green in color, as are the larvae at the time of hatching. Larvae are released into the plankton at the 3- or 4-segmented stage. They are typical spiomorphic larvae.

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[1] Fellow of the Lalor Foundation.

At the time of hatching the larvae have a well developed prototroch and telotroch, finely serrated provisional setae, strong sensory cilia on the prostomium, and two pairs of brownish-red eyes. The pygidium has four anal cirri, which look as though they contain bacillary glands.

In the later larvae gastrotrochs develop on the posterior segments, and the neurotroch appears to extend to the posterior border of the first segment. The provisional setae are replaced by the adult type and the pygidium loses the anal cirri. The prostomium and peristomium are fused to form a bell-shaped anterior end. The lateral lips of the vestibule are fused anteriorly. There are posterio-lateral palp-like projections of the vestibule wall which are capable of great extension. Diffuse light brown pigment develops in the wall of the vestibule, as well as on the prostomium and pygidium. Palps and branchiae do not develop before the 9-segmented stage. From the 10-segmented stage on, the larvae appear to be bottom dwellers. In nature metamorphosis must occur at about the 13-segmented stage, since one collection contained a specimen 13 segments long which did not possess any larval characteristics.

Electrical stimulation of light emission in fireflies. J. F. Case and John Buck.

To provide further data on the control of flashing in the fireflies *Photinus pyralis* and *Photuris pennsylvanica,* the effects of variation in strength, duration and frequency of electrical stimuli were studied. Pulses from a Grass P4 stimulator were applied either to the thoracic ventral nerve cord or directly to photogenic organs with central connections severed. The photogenic responses of both species resemble those of known neuro-effector systems in showing a well defined stimulus intensity threshold, facilitation and adaptation. Both species give evidence of complex neuro-effector organization in that areas or sub-units of the light organs may respond asynchronously to the same external stimulus.

The two species differ in spontaneous light emission, *P. pennsylvanica* producing shorter flashes which are characteristically double peaked, a small inflection occurring on the ascending limb of the major peak. This suggestion of more precise neural control of flashing by *P. pennsylvanica* is confirmed by the considerably higher stimulus frequencies to which its light organ can respond with discrete flashes (at least 20 per second compared with less than 5 per second for *P. pyralis*) and by its considerably shorter response latency to high frequency stimulus trains.

In *P. pennsylvanica* response latency to direct organ stimulation is about 70 msec. and about 200 msec. to thoracic cord stimulation. Since only a small fraction of these delays can reasonably be accounted for in terms of synaptic delay and neural transmission time it appears that the control of light emission must involve either some rather unconventional type of neuro-effector linkage or considerable delay within the photogenic tissue.

The nature of electrical responses of doubly-innervated insect muscle fibers. J. Cerf, H. Grundfest, G. Hoyle and F. V. McCann.

Depolarizing pulses applied to muscle fibers of *Romalea microptera* through one inserted microelectrode evoke responses at another (recording) microelectrode at a low level of depolarization. As the pulse strength is increased larger, spike-like responses are obtained. These are, however, always graded events. Sustained applied depolarization produces repetitive pulsatile responses, but successive potentials become smaller and may die out. The maximum magnitude of the responses is about 35 mV.

Keeping the pulse strength constant and recording at different distances from the stimulating electrode, the spike-like responses are seen to die out very rapidly. At 3 mm. distance even the largest are barely detectable. The graded responses are therefore decrementally propagated.

Junction, or postsynaptic potentials (psp's) evoked by stimulating "fast" or "slow" nerve fibers decrease with increasing depolarization of the muscle fiber. When the membrane potential is reversed to a sufficient extent, the psp's reverse in sign. With increasing membrane polarization the psp's increase in amplitude. These findings suggest that psp's are generated in electrically inexcitable membrane.

The "fast" psp's evoked during strong membrane hyperpolarization continue to produce spike-like responses. The latter often appear to be overshooting and may be initiated at lower levels of depolarization than in normally polarized fibers.

These data can account for the different types of activity evoked in one muscle fiber by stimulation of its "fast" or "slow" nerve. The psp's generated synchronously at multiple sites of innervation evoke graded, decrementally propagated pulsatile responses. Different combinations of the two response components, large "fast" psp's evoking large graded responses; small "slow" psp's producing smaller graded activity, result in the characteristically different mechanical responses of these doubly-innervated muscle fibers.

Neuromuscular transmission in the grasshopper Romalea microptera. J. CERF, H. GRUNDFEST, G. HOYLE AND F. V. MCCANN.

Some muscle fibers of insects receive double motor innervation, one fiber giving rise to large electrical responses and powerful twitch contractions, the other evoking smaller potentials and weaker contractions. This is also the case in flexor and extensor muscles of the meso- and metathoracic legs of *Romalea.* The extensor muscle of the jumping leg receives a single "fast" and a single "slow" axon which leave the ganglion in separate nerve trunks homologous to those designated 3b and 5 in locusts (Hoyle, 1955).

Intracellular recording reveals that almost all the muscle fibers receive branches from the "fast" axon, although some have been found without "fast" responses. These were fibers with particularly large "slow" responses. The resting potentials were 50–70 mV in magnitude. "Fast" responses typically involved a small overshoot. Return to the resting potential was often almost as fast as the rising phase but in many muscle fibers the later part was much slower than the first. This was attributed to the presence of persistent junction (postsynaptic) potentials. "Fast" responses are followed by a refractory period for the spike-like component.

Responses to stimulation of the "slow" nerve were found particularly in a muscle bundle situated at the proximal border of the extensor. These fibers have a richer tracheal supply than purely "fast" innervated ones. The "slow" responses ranged widely in magnitude. The smaller ones had a long time-course and summed to give a plateau of depolarization during repetitive stimulation; they showed no refractoriness. The larger ones gave rise to graded secondary responses and had a shorter time-course, though the postsynaptic potential was quite long in some cases.

The responses to both kinds of nerve stimulation were similar when recorded simultaneously at different points along the fiber, reflecting the multiterminal nature of the innervation.

Larval development of the mud crab Neopanope texana sayi (Smith).[1] NORMAN A. CHAMBERLAIN.

Larvae of *Neopanope texana sayi* have been successfully reared in the laboratory through the second crab stage. Two egg-bearing females were collected from Great Pond, Falmouth, Massachusetts, on July 22, 1957, and maintained in the laboratory. The larvae hatched as prezoeae and developed through four zoeal stages and one megalopal stage before metamorphosing into the first crab stage. The larvae were cultured in 20-liter glass jars containing 15 liters of sea water which was aerated continuously. Cultures of larvae in isolation were also maintained. The temperature of the cultures remained at 24.0° C. ± 1.0° C. All prezoeae shed within five minutes to become first zoeae. Duration of the later stages in days for the two cultures was, respectively: first zoea, 12, 8; second zoea, 3, 2; third zoea, 1, 1; fourth zoea, 2, 2; megalops, 7, 7; first crab, 7, 7. Thus the planktonic stages lasted 25 and 20 days, respectively.

Zoeal stages were fed *Phaeodactylum tricornutum* (200,000/ml.) and *Dunaliella euchlora* (20,000/ml.). Early larvae of *Arenicola cristata* were fed to the zoeal and megalopal stages with the exception of the first zoeae of the first culture which were fed only *Phaeodactylum tricornutum* for the first six days of their development. This fact probably accounts for the observed difference in the durations of the first zoeal stages. Megalopal and crab stages were fed *Artemia salina* nauplii. Crab stages were also fed small pieces of clam gill and mantle.

These data indicate that duration of planktonic stages can be shorter than has been estimated from plankton studies.

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[1] This study was aided by a summer fellowship from the Woods Hole Oceanographic Institution.

Fertilizability of Arbacia eggs after pretreatment in trimethylated xanthine derivatives. RALPH HOLT CHENEY.

A viability time table for *Arbacia punctulata* gametes in relation to time was noted by the author in 1950. The effect of $M/400$ di- and trimethylated xanthines was published in 1956. Concurrently with a study of inhibitory effects of derivatives of 2:6 dioxypurines on mitosis and growth of this species, the insemination process and appearance of the fertilization membrane were observed.

Eggs were shed into equal volumes of sea water, and into $M/200$ and $M/400$ concentrations in SW of trimethylated xanthine (caffeine), 8-methoxycaffeine, 8-ethoxycaffeine, and 8-chlorocaffeine. Eggs were mixed with non-treated, fresh sperm in SW after eggs were pretreated for 4, 10, 24, and 48 hours.

Delay in insemination time and appearance of the fertilization membrane was only slight unless the egg pretreatment period exceeded four hours although a 30-minute immersion does affect subsequently the mitotic process and early growth. Order of increasing delay or complete inability of the egg to produce the FM after 10, 24, and 48 hours in $M/200$ or $M/400$ showed the same sequence; namely, 8-MOC; 8-EOC; C; and 8-CC.

All of these compounds have been reported to penetrate the plasma membrane readily in plant cells but only EOC penetrates the nuclear "envelope." Centrifugation studies by the author (1949) demonstrated an egg surface-action effect of the trimethylated xanthine. Unpublished data with respect to sperm potency after subjection to these compounds indicate that the egg remains normal longer than sperm in identical molarities. It is suggested that the variation of the physico-chemical surface forces induced by these compounds may account for the differences in the insemination time-period and the lifting of the fertilization membrane.

Dioxypurine derivatives as mitotic and growth inhibitors. RALPH HOLT CHENEY.

Dimethylated (theophylline) and trimethylated (caffeine) 2:6 dioxypurines, and a methoxy-group (OCH_3) substituted for H at C^8 (methoxycaffeine), an 8-ethoxy radical (OCH_2CH_3) the 8-ethoxycaffeine, and a Cl substitution 8-chlorocaffeine, were employed in $M/200$ (5 mmol/L) and $M/400$ (2.5 mmol/L) concentrations. Test cell was the egg of *Arbacia punctulata*. Non-treated, fresh sperm were mixed in sea water (SW) with eggs pretreated 30 minutes in the experimental solutions: SW alone, TpSW, CSW, 8-MOCSW, 8-EOCSW, and 8-CCSW.

Based upon (1) degree of delay and induced abnormalities of the mitotic process and cell division, and (2) observation of the maximal growth at death in equivalent molarities, the order of increasing inhibitory effectiveness of the number, position, and nature of the radicals was determined. In $M/200$, the order of increasing effectiveness indicated by relative mitotic inhibition was Tp; 8-MOC; 8-EOC; C; 8-CC. An identical sequence was demonstrated by the death series. Using $M/400$, a similar order was noted except that the mitotic inhibition results suggested a possible reversal in the relative effectiveness of 8-EOC and C.

Analyses of sequences demonstrate an inhibitory increase associated with an increase in N-bound methylation, still greater inhibition with ethoxy- radical compared with the methoxy-group, and a maximal effect with the chlorine substitution for H at C^8. Relative inhibitions indicate a significant relationship between the molecular structure involving CH_3, OCH_3, OCH_2CH_3, and Cl, the relative penetration power of these compounds at the nuclear border, and possibly the electro-negative property of chlorine. Results regarding the relative inhibitory effect of C and 8-CC are in general agreement with the report regarding these same dioxypurine derivatives upon plant cells in *Allium* root tip. See Kihlman, B. (*Symb. Bot. Upsal.,* **11**, No. 2: 1–40 (1951); **11**, No. 4: 1–96 (1952)).

The demonstration of histamine in heparin-containing invertebrate cells.[1] ALFRED B. CHAET AND WILLIAM R. CLARK, JR.[2]

This study of invertebrate eggs resulted from a consideration of the mammalian mast cell which has been shown to contain heparin and more recently to be a major source of histamine.

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[1] Supported in part by Parke, Davis Company.

[2] Lederle Medical Student Research Fellow.

There exists the possibility that a single substance within the cell binds both heparin and histamine, and the work, *in vivo* and *in vitro,* of other investigators suggests that there may in fact be a heparin-histamine complex. With these studies in mind, it seemed worthwhile to search for histamine in other cells known to contain heparin. Since blood clotting experiments and/or the metachromatic reaction have indicated the presence of heparin in eggs of the clam (*Spisula solidissima*), the sea urchin (*Arbacia punctulata*), and the annelid (*Chaetopterus pergamentaceus*), we assayed these eggs for histamine using the micro-chemical technique of Lowry *et al.* The results suggest that these eggs are rich in histamine. Our analyses show that the Spisula egg contains 2.0×10^{-6} μgm histamine base per egg and that 4.8×10^{-6} μgm histamine is present in Arbacia. Preliminary experiments indicate 9.6×10^{-6} μgm histamine per Chaetopterus egg. These figures are slightly lower than those calculated by others for rat mast cells (6.0×10^{-6} μgm histamine per cell). Disruption of the cell membrane is apparently sufficient to liberate most of the histamine within the egg since homogenization, freezing and thawing, or hypotonic solutions result in histamine release. Since compound 48/80 releases histamine from mast cells *in vivo,* we have attempted to liberate histamine from Spisula and Arbacia eggs using various concentrations of 48/80 (20–200 mg%); however, in no case did it act as a liberating agent. These experiments are complicated by the fact that 48/80 interferes with the histamine determination. Further studies on the mechanism of histamine release by histamine liberators, as well as by heparin liberators, are being carried out.

A technic for preparing whole mounts of veliger larvae. A. C. CLEMENT AND J. N. CATHER.

Excellent, almost life-like whole mounts of the young veliger larvae of *Ilyanassa obsoleta* and *Anachis avara* have been prepared by the following relatively rapid and simple technic. Larvae are first immobilized by placing them in a mixture of one part saturated aqueous solution of chloretone and two parts sea water. Three or four minutes exposure to this mixture is adequate; prolonged exposure may be damaging. The larvae are then fixed for one hour in a solution of 10% formalin in sea water. In a satisfactory proportion of the cases the fixed larvae will show the velum fully expanded. The larvae are next dehydrated in ethyl alcohol (10 minutes in each of the following: 35%, 70%, 95%, and two successive baths of 100%) and mounted in Euparal under coverslips supported by strips of filter paper. In larvae in which the yolk has been largely or wholly absorbed, structural details of the digestive tract, velum, foot and other parts may be seen with great clarity in these preparations. The shell and velar cilia are well preserved. The natural pigments are preserved initially, but some deterioration of the velar pigment has been observed after a month. A light tinting of the tissues with Orange G in 95% alcohol enhances the value of the preparations for some purposes and provides a pleasing contrast with the natural pigments.

The aminopeptidase and catheptic activity of the egg of Ilyanassa obsoleta. J. R. COLLIER.

The activities of two proteolytic enzymes, leucine aminopeptidase and cathepsin C, were identified in the egg of *Ilyanassa obsoleta.* These enzymes were characterized by 1) their specificity toward synthetic substrates, 2) their pH optimum, and 3) their requirements for activation.

The aminopeptidase activity was determined by measuring the ammonia liberated after the enzymatic hydrolysis of leucinamide. At a substrate concentration of 0.05 *M,* optimal hydrolysis of leucinamide occurred at pH 8.2. The addition of 0.02 *M* $MnCl_2$ (final concentration) gave a three-fold increase in the hydrolysis of leucinamide. Leucine aminopeptidase is the only enzyme known to hydrolyze leucinamide under these conditions.

The activity of cathepsin C was identified by the fact that the *Ilyanassa* egg hydrolyzes glycyl-1-tyrosinamide at the amide bond. Fruton has shown that this substrate is hydrolyzed only by chymotrypsin and, at an acid pH, by an intracellular proteinase which he has designated as cathepsin C. At a substrate concentration of 0.05 *M,* optimal hydrolysis of glycyl-1-tyrosinamide by the *Ilyanassa* embryo occurred at pH 6.0. This enzyme was not activated by 0.01 *M* cysteine. A second pH optimum, at pH 7.5, may be due to the activity of an enzyme similar to chymotrypsin.

The activity of both leucine aminopeptidase and cathepsin C showed a marked increase during the course of embryonic development. This increase in activity occurred at about the fourth day of development. The relative activity of aminopeptidase was found to be about 25 to 30 times greater than the activity of cathepsin C.

Egg membrane lysis by a sperm extract in Hydroides hexagonus (Annelida).[1] ARTHUR L. COLWIN AND LAURA HUNTER COLWIN.

Eggs of *Hydroides hexagonus* were immersed in solutions of sea water extracts of frozen-thawed sperm of the same species. The principal substance of the vitelline membrane appeared to swell and then dissolve; a very thin inner portion appeared to remain as a capsule close to the egg; a very thin outer portion became elevated from the egg and was frequently ruptured and shed. Possibly the outer portion represents the *outer border layer,* or some part of that layer, which can be seen in electron micrographs of thin sections. Following treatment, the outer portion can be caused to wrinkle and often some part comes to lie directly against the inner capsule. However, if a treated egg is compressed, globules of exudate will pour out through the inner capsule and push the outer portion away. In cases where the outer portion has been shed, eggs may be pushed together so that the inner capsule of one egg directly touches the inner capsules of adjacent eggs. These facts are interpreted as indicating that the substance between the inner and outer portions of the vitelline membrane becomes liquefied.

Essentially the same results were obtained with fertilized and with unfertilized eggs.

It is concluded that the sperm of *Hydroides hexagonus* contains a lysin or lysins which can dissolve the principal material of the vitelline membrane but cannot attack the inner and outer portions. This conclusion lends strength to the recently expressed view that the individual spermatozoon of *Hydroides hexagonus* exerts lytic action at the site of its passage through the vitelline membrane (Colwin, Colwin and Philpott, 1957).

Observations of sperm entry during re-fertilization in Saccoglossus kowalevskii (Enteropneusta).[1] LAURA HUNTER COLWIN AND ARTHUR L. COLWIN.

In this egg normal sperm entry is marked by the formation of a prominent fertilization cone (Colwin and Colwin, 1954). After the fertilization membranes and other surrounding layers had been dissected away from fertilized eggs, fresh insemination led to the entry of additional spermatozoa into these eggs. Re-fertilization was observed to occur as late as the early blastula stage. The entry of these additional spermatozoa is also marked by formation of a cone or cone-like structure, but this structure is less prominent than the fertilization cone of normal sperm entry.

Spermatozoa also were observed to enter artificially activated, but unfertilized, eggs provided the membranes had been dissected away. Cones similar to those observed in re-fertilization were formed.

The cortical response of the Nereis ovum to activation after centrifuging.[2] D. P. COSTELLO.

If uninseminated eggs of *Nereis limbata* are centrifuged at 6000 to 200,000 G for appropriate periods of time (60 minutes for low or moderate, 10 minutes for higher accelerations) and inseminated shortly after removal from the centrifuge tubes, an asymmetrical perivitelline space is produced and asymmetrical jelly outflow occurs. Since the eggs must be "cushioned" during the centrifuging, in isopycnotic sucrose, this is washed from the eggs before inseminating. The asymmetry of the cortical response of the centrifuged egg is obviously due to displacement of the jelly-precursor granules, which accumulate at the centrifugal pole of the egg, to a degree dependent upon the intensity and duration of the applied centrifugal force. Parthenogenetic agents produce the same asymmetry of cortical response in the strongly centrifuged eggs, as do spermatozoa.

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[1] Supported by a grant (RG-4948) from the National Institutes of Health, U. S. Public Health Service.

[2] Aided by a grant from the National Institutes of Health, RG-5328.

If unfertilized Nereis eggs are centrifuged sufficiently to sediment the cortical jelly-precursor granules, and then treated with alkaline NaCl at pH 10.5, the vitelline membranes elevate to an exaggerated degree, with a marked asymmetry. The perivitelline space formed is widest at the centrifugal pole and lacking at the centripetal pole. With continued exposure to the alkaline NaCl, the jelly in the asymmetrical perivitelline space swells, forcing the egg against the centripetal region of the vitelline membrane. Eventually the membrane ruptures at this point, and the egg is extruded; it invariably emerges with the centripetal oil cap forward, due to the continued pressure of the swelling jelly in the wide centrifugal region of the perivitelline space.

This provides further evidence that the cortical response to activation in the Nereis egg is basically similar to that in the echinoderm egg.

Electrical activity of supramedullary neurons of puffer (blowfish) Sphcroides maculatus. S. M. Crain, M. V. L. Bennett and H. Grundfest.

Supramedullary neurons of puffer, 300–500 μ in diameter, are readily visualized and penetrated with microelectrodes. Their resting potentials range to 70 mv. Spikes up to 100 mv. in amplitude and 3–5 msec. in duration are evoked *directly* by stimulation with an intracellular electrode or with external electrodes close to the cell, and *indirectly* by scratching the skin, or by electrical stimulation of spinal cord, dorsal (but not ventral) roots, or cranial nerves. Indirect spikes have an inflection on their rising phase at 20–30 mv. depolarization, which is also threshold for direct spikes. During refractoriness or hyperpolarization with another intracellular electrode, indirect stimuli may fail to produce full-sized spikes, failure occurring at the inflection. The remaining small potential cannot be graded by varying stimulus strength.

When recording extracellularly close to the cell, electrode negativity indicates inward current flow and active membrane under the electrode; positivity denotes outward flow and active membrane distant from the recording site. At the supramedullary cell's dorsal surface, extracellular recording reveals a large positivity associated with the first component of the indirect spike and variations from small positivity to large negativity associated with the bigger second component. Therefore, the active membrane producing the second component neighbors or includes the cell's dorsal surface; that producing the first is distant.

As the interval is shortened between a direct and a succeeding indirect spike, the second component of the latter fails to fire. The first component, although reduced in size during the observed post-spike reduction of membrane resistance, cannot be abolished. Moreover, it decays with a time constant greater than that of the membrane. These data (see also abstract by Bennett, Crain, and Grundfest in this issue) suggest that the first component of the indirect spike is a postsynaptic potential.

The inhibition by a series of nitro- and halophenols of glucose-6-phosphate dehydrogenase from Arbacia eggs and yeast. Robert K. Crane, Howard H. Hiatt and G. H. A. Clowes.

The influence of a series of substituted phenols on the activity of glucose-6-phosphate dehydrogenase from Arbacia eggs (A) and yeast (Y) and of 6-phosphogluconate dehydrogenase from Arbacia eggs (PGD) was determined with the usual spectrophotometric techniques. The enzymes differed greatly in the degree to which they were inhibited. In general, A was strongly inhibited at low concentrations of certain phenols; Y and PGD were inhibited to a lesser extent at much higher concentrations. The data suggest the possibility that the inhibition of A differs in character from the inhibitions of Y and PGD. This possibility is being explored with enzyme preparations from rapidly dividing cells other than Arbacia eggs. Also, the relative inhibition of A by the individual phenols shows a pattern markedly different from that previously established for inhibition of cell division and of aerobic phosphorylation. It will thus be possible to investigate the respective roles of glucose-6-phosphate oxidation and of aerobic phosphorylation in cell division and other cellular processes. The data from the present experiments are recorded below. The numbers given for each of the enzymes, A, Y, and PGD, are the per cent of inhibition which occurred at the stated concentration of substituted phenol. The compounds are listed in descending order of their inhibition of A. 2,4,5-trichlorophenol, 0.000066 *M*, A-55, 0.00033 *M*, A-86, Y-0, PGD-0; 4,6-dinitro-o-cresol, 0.00017 *M*, A-58, Y-0, PGD-6, 0.0003 *M*, A-80, Y-16, PGD-29, 0.000033 *M*, A-19, Y-0, PGD-0; 2,4-dinitro-o-cyclohexylphenol, 0.0002 *M*, A-70, Y-17, PGD-18; 2,6-dinitro-4-chlorophenol, 0.00033 *M*, A-73, Y-0; 2,4-dichlorophenol, 0.00033 *M*, A-47, Y-0; 2,4,6-trinitrophenol, 0.00033 *M*, A-37, Y-0; 2,4-dinitrothymol, 0.00033 *M*, A-23, Y-0, PGD-0; 2,4-dinitrophenol, 0.00033 *M*, A-13, Y-0, PGD-7; 4,6-dinitrocarvacrol, 0.00033 *M*, A-11, Y-0, 0.001 *M*, A-54, Y-0, PGD-28; 2,6-dinitrophenol, 0.00033 *M*, A-8, Y-16, PGD-0, 0.0017 *M*, A-72, Y-16, PGD-15; p-nitrophenol, 0.00066 *M*, A-18, Y-0, PGD-0; o-nitrophenol, 0.002 *M*, A-55, Y-57, PGD-49.

Preliminary studies on the incorporation of glucose-U-C^{14} into the polysaccharide of Arbacia and Mactra larvae and its inhibition by 4,6-dinitro-o-cresol. ROBERT K. CRANE, ANNA K. KELTCH, C. PATRICIA WALTERS AND G. H. A. CLOWES.

Twenty-four-hour swimming forms of Arbacia and of Mactra were incubated in sea water containing added glucose-U-C^{14}. After an initial period of incubation, various concentrations of 4,6-dinitro-o-cresol were added to some of the vessels and the incubation was continued. At termination of incubation, the larvae were recovered by centrifugation and their acid-soluble components were separated by paper chromatography. The two-dimensional solvent system of Bandurski and Axelrod was used. The areas of the developed papers containing C^{14} were identified by contact autoradiography. Three or four well-defined spots, which have not been identified, could be made out. However, the greatest amount of radioactivity was found to remain at the origin, suggesting that significant incorporation into polysaccharide had occurred. The radioactivity of this spot was markedly less when 4,6-dinitro-o-cresol had been added. Polysaccharide identification of this spot was not attempted. Instead, "glycogen" was isolated from duplicate samples of larvae by the conventional alkaline digestion-ethanol precipitation procedure. Assay of this "glycogen" for radioactivity by the usual counting techniques and for total carbohydrate by the anthrone method revealed that glucose-U-C^{14} was indeed incorporated during incubation. The specific activity of the "glycogen" in the control larvae increased during prolonged incubation. In the "glycogen" of larvae to which 4,6-dinitro-o-cresol had been added, the specific activity decreased. The experiments were not completed, owing to the scarcity of viable eggs, and they will be continued next season.

The preservation of intact erythrocytes of marine vertebrates for blood group research.[1] J. E. CUSHING, G. J. RIDGWAY AND G. L. DURALL.

The glycerol-freezing technique (*cf.* Chaplin *et al.*, 1954) appears to be a promising method for preserving the erythrocytes of marine vertebrates collected in the field. The following points serve as a guide for further study. Cells of several species of fish and one humpback whale have been treated. Whole blood from vessels in the gill, tail or other areas is allowed to run into screwcap bottles, as much as 25 to 50 ml. being taken. An equal volume of a solution containing glycerol (40%) and 5% trisodium citrate (60%) is added and the mixture placed in a freezer (approximately −20° C.) after thirty minutes.

Cells are recovered from a few ml. aliquots of thawed blood by reducing the glycerol content of their milieu stepwise at five-minute intervals, using cool solutions. For example, albacore cells were recovered by first adding an excess of 20% glycerol in 2% NaCl and then reducing the glycerol, by dilution with 2% NaCl, to 14, 8.5, 6.5, 5.0, 4.0, 3.0, 2.0 and 1.5 per cents; 1.5% NaCl was substituted and the glycerol reduced to 0.7, 0.35 and finally 0.0%. (Reasonable deviations from this schedule were also successful.) The cells obtained kept well and could be used in agglutination and absorption studies.

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[1] Supported in part by the Penrose Fund, American Philosophical Society, and the Office of Naval Research.

While the degree to which different antigens remain unchanged during prolonged storage probably varies, as in humans, cells of the whale, shiner seaperch, goosefish and three species of salmon retained antigenic specificities during preservation. Absorption experiments suggest individual differences in the antigens of preserved albacore cells that appear to resemble those of other tuna.

The effect of nervous system extracts on inhibition and excitation in single nerve cells. C. R. ELIOT, A. KAJI, P. SEEMAN, E. UBELL, S. W. KUFFLER AND A. S. V. BURGEN.[1]

Stretch receptors in lobster and crayfish contain a sensory neuron on whose dendrites an efferent nerve fiber forms inhibitory synapses. On this system extracts from acetone-dried lobster nerve cords and leg nerves were tested. First the physiological action of crude extracts and then of chromatographically purified fractions was determined. The following effects on the nervous structures were studied:

1. Change of discharge rate, largely reflecting the state of the dendrites of the sensory cell.
2. Changes in resting potential and in inhibitory synaptic potentials.
3. Changes in effectiveness of inhibitory transmission.

Three distinct kinds of action were seen, namely, slowing (inhibition) of sensory discharge, acceleration of sensory discharge, and reduction in effectiveness of inhibitory transmission. Each of these effects could be obtained separately from crude extracts, depending on concentration and source of extracts, leg nerve extracts having little inhibitory effect on the discharge rate. In chromatographic fractions run in phenol-ammonia-water or in chloroform-methanol-HCl, the fractions with relatively low mobility mainly blocked inhibitory transmission and slightly depolarized the nerve cell. The most mobile fractions had a purely excitatory effect. Some of the intermediate fractions inhibited the sensory discharge in a manner similar to gamma-aminobutyric acid, causing mainly a small hyperpolarization or no appreciable membrane potential changes. The activity of the excitatory material decreased after acid hydrolysis.

The mating type system in variety nine of Tetrahymena pyriformis. ALFRED M. ELLIOTT AND GORDON M. CLARK.[2]

Matings involving parental clones TC 105, TC 110, TC 156, TC 160, TC 148, and TC 89 were used, as well as their F_2, F_3, F_4, and F_5 progeny. This variety differs from the other varieties studied so far in that (1) the old macronucleus migrates anteriorly after nuclear exchange has occurred, (2) conjugation is delayed for 18 to 36 hours after cultures have been mixed (25° C.), (3) no detectable immaturity period exists, and (4) this variety has only been found in the tropics to our knowledge.

The lack of an immaturity period complicates the picture in that no check can be made as to whether nuclear exchange has occurred. Since pairs are isolated when 70–80% are in the anlagen stages, it is felt that a high percentage of cultures obtained are from pairs in which true nuclear exchange has occurred. Variety nine possesses five mating types. No new ones have been derived. A cross of any two mating types yields either of the parental types in 98% of the cases. Mating types other than the parental, selfing caryonides, and non-reactive clones make up the remaining 2%. Non-reactive clones may possess an immaturity period the length of which may be genetically determined. These clones might be used in the future to obtain clones with an immaturity period to serve as a check as to whether nuclear exchange has occurred, which also can be checked by inbred lines using the serine mutant as a marker. Cytoplasmic exchange occurs but may not be a universal phenomenon in the variety. Time of separation of the pairs, after the anlagen stages are reached, is highly variable. The data to date would support a mating type system analogous to the B-system of *Paramecium aurelia* with multiple mating types involved.

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[1] Fellow of the Lalor Foundation.

[2] This investigation was supported in part by a research grant (PHSE 1416) from the National Institutes of Health, Public Health Service.

X-radiation effects during conjugation of Tetrahymena pyriformis. Alfred M. Elliott and Gordon M. Clark.[1]

An attempt was made using x-rays to induce back mutations to the serine and pyridoxine requirement. Conjugating F_3 progeny (var. 9) serine non-requirers and conjugating F_3 progeny (var. 2) pyridoxine non-requirers were x-radiated during early prophase. If no back mutation occurred, the serine F_4's should not require serine and the pyridoxine F_4's pyridoxine. Dosages from 200 kr–600 kr at 100-kr intervals were used (dose rate 4720 r per minute). Forty pairs were isolated into peptone for each cross and for each dosage, with half the pairs incubated at 11° C. for 24 hours and then returned to 25° C. The remainder were incubated at 25° C. Suitable controls were used. Clones obtained were checked for growth on peptone, complete, pyridoxine and serine-deficient media.

Vegetative cells survived dosages of 600 kr as opposed to 300 kr for conjugants. No significant differences in survival were observed for pairs incubated at 11° C. or 25° C. In the pyridoxine cross viability was 17.7% for controls, 27.5% for 300 kr and 40% for 200 kr. The serine control gave viabilities of 62.5%, 12.5% at 300 kr and 25% at 200 kr (pooled data for both temperatures). Serine conjugants are more sensitive to x-radiation than the pyridoxine ones. The apparent higher viability in the x-rayed versus the control pyridoxine cross may be due to separation of pairs before nuclear exchange has occurred. From the pyridoxine work 27 irradiated clones were obtained. Eighteen grew without pyridoxine, six required and three would grow only on peptone. In the serine study 15 clones were obtained, 12 of which were serine non-requirers, two gave variable results and one would grow only on peptone.

Prophase is a radiosensitive stage in conjugating forms with bridges and fragments observed at anaphase stages.

Post x-radiation effects of temperature on vegetative cells of Tetrahymena pyriformis. Alfred M. Elliott and Gordon M. Clark.[1]

Variations exist in the x-ray sensitivity for the various varieties of *Tetrahymena*. Strains in varieties 1, 2, 5 and 6 survive 500 kr and 3, 4, 7 and 8 stand 400 kr. Strains TC 89 (serine mutant) will not survive above 300 kr although inbred F_4 lines survive 600 kr.

Using starved cultures of TC 89 (var. 9), the effects of various post x-radiation temperatures on fission rate and recovery after incubation at 11° C., 15° C., 20° C., 25° C., 32° C. and 35° C. were studied. Dosages of 100, 200, and 300 kr were employed (dose rate 4720 per minute). Forty vegetative cells were isolated onto peptone (25° C.) and incubated at the various temperatures for 24 hours and then returned to 25° C. Suitable controls were used.

A stimulatory effect of 100 kr, as reflected by an increased rate of fission over controls, was seen at all temperatures. Above 100 kr suppression of fission by x-rays increases with increasing dose. Survival of cells after x-radiation is somewhat higher at lower temperatures, with variable results at 11° C. and 20° C. No irradiated cells survived a post-irradiation temperature of 35° C. For those cells surviving the higher temperatures, recovery was more rapid and seemingly temperature-dependent, increasing with increasing temperatures.

Abnormal cells, characterized by budding and blistering of the pellicle, as well as fusion of fission products to form multinucleated masses, were common at 300 kr. A delay in appearance of these forms was evidenced at lower temperatures. Incubation for longer periods of time after x-radiation at various temperatures may produce in the future studies with more striking results.

The effects of some amino acids on the perfused lobster heart. P. E. S. Enger and A. S. V. Burgen.[2]

The response of the perfused heart of the lobster *Homarus americanus* to various amino acids has been tested. Aspartate and glutamate were both found to have a marked stimulant

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[2] Fellow of the Lalor Foundation.

effect on the rate and amplitude of the heart beat, effective down to a few micrograms. Aspartate had a greater action on the rate than had glutamate. Both substances in higher concentrations produced an increase in diastolic tone. Asparagine was ineffective, but glutamine showed a very weak stimulatory action possibly due to contamination with traces of glutamate. L and D glutamate were equally effective. A powerful inhibition of the heart was produced by γ-aminobutyrate. Usually this action was purely on rate but occasionally some depression of force of contraction was also produced. A similar but weaker effect was produced by β-guanidopropionate, β-alanine, γ-guanidobutyrate, δ-aminovalerate, and δ-guanidovalerate in descending order of activity. No significant response was obtained with glycine, alanine, leucine, serine, lysine, arginine, cystine, methionine, tyrosine, histidine, hydroxy-proline, β-aminobutyrate, ω-aminooctanoate, or the peptides diglycine, tryglycine, and carnosine. Aqueous alcohol extracts of lobster nerve cord and leg nerves produced regular stimulation of the heart rate and amplitude and in some dilutions a transitory inhibition. Preliminary separation of these two types of activity was obtained by paper chromatography with a phenol-water-ammonia system. These fractions were also tested on the segmental stretch receptors of the lobster and crayfish.

Electron microscope studies of the flagella of Chlamydomonas. SARAH P. GIBBS, DELBERT E. PHILPOTT AND RALPH A. LEWIN.

Ultrathin sections of *Chlamydomonas moewusii* were studied to determine normal flagellar structure, the structure of the flagella in paralyzed mutants, and the nature of the protoplasmic bridge formed between mating cells. Each flagellum reveals the characteristic 9 double tubular filaments surrounding a central pair, all imbedded in a structureless matrix and enclosed by a thin sheath continuous with the plasma membrane of the cell. The two central fibrils extend slightly beyond the peripheral ones, forming a mucronate tip. Slightly distad from the cell surface, a diaphragm traverses the flagellum, and below this the 9 peripheral fibrils continue to form the walls of the basal body (blepharoplast). Extending slightly above and below the diaphragm, there is a short cylinder (possibly two appressed half-cylinders) of osmiophilic material in the center of the flagellum and the blepharoplast. The central fibrils appear to end just above this cylinder. In this species of *Chlamydomonas* the two flagella arise from opposite sides of an anterior papilla and the blepharoplasts are continuous basally. Anteriorly the fused basal bodies send forward a conical median projection into the papilla.

The flagella of five paralyzed mutants were found to be identical to normal flagella in all respects. Two mutants had stubby flagella, 2–4 μ instead of 12–24 μ long, but otherwise normal in structure. Two mutants which through the light microscope appear flagella-less were found to have very short flagella 0.6–1.0 μ long. Their structure is under investigation.

Sections of mated pairs show that the intergametic bridge is formed by an extension of the anterior projections of the blepharoplasts. Whether the anterior projections from both cells grow out and fuse with each other, or whether only one grows out, invades the other cell, and connects to its blepharoplast, is presently being studied.

Effect of sodium fluoride on the development of Arbacia punctulata. SYLVIA S. GREENBERG AND ARTHUR GREENBERG.

Fluoridation of community water supplies, as a caries preventive measure, is a widespread practice. The optimum concentration of sodium fluoride has been determined to be one to two parts per million. Hypotheses have been advanced as to the nature of the chemical reaction between the fluoride ion and bone and tooth structure. Most of the experimental work has been done with rats and hamsters fed increasing concentrations of fluoridated water. Subsequent sectioning and ashing of hard tissues have indicated the extent of incorporation of fluoride ion. In the present experiments, the eggs of the sea urchin, *Arbacia punctulata,* were fertilized and allowed to develop in sea water containing varying concentrations of sodium fluoride. Skeletal changes in the developing plutei were followed. At concentrations of one and ten parts per million, differences between experimental and control animals were not detectable with the light microscope. At fifty parts per million, approximately 60% of the animals exhibited skeletal aberrations. These included plutei with one arm, no arms, partially developed arms, as well as completely skeletonless bodies. At seventy-five parts per million, most of the cultures were entirely free of skeletal spicules. When these amorphous forms were placed in non-fluoridated sea water, skeletal development was stimulated in two days.

The intercellular cement of sodium fluoride-treated embryos was noticeably weakened at concentrations of fifty and seventy-five parts per million. Free blastula cells were present as well as exogastrula forms. The affected embryos resembled those grown in calcium-free sea water. The reduction in adhesiveness was not as great, however, since many animals were able to develop to more advanced stages. The data suggest that high concentrations of sodium fluoride in the growing media remove available calcium and phosphate ions, which are necessary for normal development of *Arbacia punctulata* embryos.

Stimulation of the taste receptors of the rat with organic salts. C. W. Hardiman.

The responses of the chemoreceptors of the tongue were studied by integrating the spike potentials elicited in the chorda tympani nerve bundle. A constant area of the tongue was bathed with the various stimulating solutions.

The concentration/response curves were determined for a number of organic salts. The response increases as the concentration increases until 0.5 *M*; the response is then almost maximal and doubling this concentration gives only a slightly larger response. The concentration at which the maximum or saturated response level occurred varied with the anion within a given cation series. The order of the ability of the anions to stimulate within a sodium series was: chloride $\geq$ oxalate $\geq$ citrate $>$ tartrate $>$ succinate $>$ formate $>$ salicylate acetate $>$ glutamate $>$ propionate $>$ butyrate $>$ oleate. The response curves of many of these same anions were determined using Li, NH_4, Mg, and K. The relative order of the anions remained the same within each of the cation series although the cation species determines to a great degree the magnitude of the maximum response.

An enhanced response of NaCl occurred after an application of lithium citrate. This enhancement was time-dependent, in that the response of NaCl shortly following lithium citrate was much greater, whereas it declined to the standard response within a few minutes. Other citrate salts did not show this enhancement, nor did oxalates or mono-sodium glutamate.

Potassium benzoate below 0.25 *M* did not stimulate the salt receptor, but indeed inhibited the small amount of spontaneous background activity. As the solution was washed from the tongue, there was a large response to water. Concentrations above 0.25 *M* responded as a typical salt. Neither KCl nor other benzoate salts produced this effect.

The spectral energy curve of Cypridina and other luminous organisms. E. N. Harvey, A. M. Chase and W. D. McElroy.

Thanks to the loan of a spectrophotofluorometer by the American Instrument Company, spectral energy curves for the bioluminescence of colorless perfectly transparent aqueous solutions of *Cypridina* luciferin and luciferase have been recorded with an accuracy of ± 5 mμ. The maximum emission of *Cypridina* is at 470 mμ and extends from 410 to 620 mμ. The luminous bacterium, *Achromobacter fischeri,* has a maximum at 495 mμ and extends from 440 to about 600 mμ. Two new strains of luminous bacteria, isolated at Woods Hole from flounder and from squid, have essentially the same spectral distribution. The ctenophore, *Mnemiopsis leidyi,* squeezed through cheesecloth and with water added to the transparent suspended material, luminesced with a maximum at 490 mμ and its emission extended from 430 to 610 mμ. A pale yellow solution of luminol plus H_2O_2 excited to luminesce with catalyst A exhibited an emission with a maximum at 460 mμ and extending from 380 to 610 mμ. All curves are slightly unsymmetrical, with the maximum nearer the blue than the red end of the emission spectrum.

Neuromuscular transmission in Limulus. G. Hoyle.

The electrical and mechanical events associated with neuromuscular transmission have been studied particularly in the closer muscle of the claw of the walking legs, with the aid of intracellular recording electrodes. The mean resting potential is about 50 mV. This muscle is innervated by both a "slow" and a "fast" nerve fiber. The mechanical response to single excitation of the former is very small. At frequencies of about 50 per second a smooth tonic contraction results. The electrical response consists of a small junction potential. This shows a moderate degree of facilitation. At about 50 per sec the maximum height is about 5 mV depolarization. The duration is about 50 msec.

The mechanical response to stimulation of the "fast" nerve fiber consists of a sharp twitch, though this does not completely close the claw. The electrical responses to a single shock vary in different fibers from about 2 to 8 mV. Their duration is about 50 msec. The smaller ones have the characteristic shape associated with a pure postsynaptic potential. Some of the larger ones show a departure; there is a small, secondary response of about 4–5 mV magnitude.

The responses show no refractoriness. During repetitive stimulation there is a small amount of growth (facilitation) in the "fast" responses. They summate completely. If they are sufficiently closely spaced the fifth or sixth may give rise to a secondary response which reaches or just overshoots the zero baseline.

The major part of the response thus consists of a small synaptic potential which nevertheless gives rise to a twitch contraction. Repetitive stimulation can evoke secondary, graded electrical responses which possibly cause an augmentation of the mechanical response.

Comparative distribution of radioactive alloxan, thiocyanate, and urea in islet and other tissues of the toadfish (Opsanus tau). ARNOLD LAZAROW AND S. J. COOPERSTEIN.

As part of a project designed to determine the mechanism by which alloxan selectively kills the insulin-producing cells of the islets of Langerhans, we have studied the uptake of radioactive alloxan by islet and other tissues of the toadfish. Since the radioactivity of the various tissues measured includes the radioactivity of contained blood, extracellular fluid, and intracellular fluid, we have attempted to differentiate between these components by comparing the distribution of radioactive alloxan with that obtained for sodium thiocyanate and urea. Sodium thiocyanate is distributed throughout the extracellular fluid, but it does not enter the cells. On the other hand, urea is distributed throughout both the intracellular and extracellular phases.

The data obtained to date indicate that alloxan and thiocyanate have similar patterns of distribution in the various tissues (with the exception of kidney) at all times following injection. On the other hand the amount of urea present in the various tissues is much greater than that observed for either thiocyanate or alloxan. Within 2–5 minutes after injection, the amount of alloxan and thiocyanate present in the islet tissue represents about 20% of the blood value. However, within 5 minutes after injection, the amount of urea in islet is equal to 62% of the blood value. By the end of 30 minutes, both the alloxan and thiocyanate have reached a value equal to 42% of blood whereas the urea reached a value equal to 85% of blood.

These preliminary results suggest that the injected alloxan is distributed primarily in the extracellular fluid. However, further work is necessary before any final conclusions can be drawn. If this observation proves to be correct it would suggest that alloxan may produce its effect by acting on a system at the surface of the beta cell.

A further study on the induced furrowing reaction in Arbacia punctulata. DOUGLAS MARSLAND[1] AND WALTER AUCLAIR.

Premature furrowing, starting as early as 40 minutes ahead of normal schedule (at 20° C.), can be induced when fertilized eggs are centrifuged for 2–5 minutes at high force (40,000–50,000 G) and at high pressure (6,000–12,000 lbs./in.^{2}).

The induction of the furrowing reaction appears to depend upon the centripetal displacement of material derived from, or affiliated with, the nucleus, as is indicated by the following considerations.

1) Eggs treated prior to prophase of first cleavage never display induced furrowing unless the nuclei are broken by the treatment. Conversely, when the nuclei are broken, induced

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[1] Work supported by the National Cancer Institute, Grant Series C–807.

furrowing always occurs in a considerable number (5–95%) of the eggs, starting some 3–6 minutes after the treatment.

2) The unbroken nuclei, in the centrifuged non-pressurized controls, come to lie in the hyaline zone, close to the oil cap, but in cells with broken nuclei, one finds 1–3 relatively small Feulgen-positive fragments lying in or near the mitochondrial zone. No trace of the other nuclear material, which presumably is thrown in a centripetal direction, has been found. Asters and spindles are not found when furrow induction precedes the first mitosis.

3) The plane of induced furrows is always at right angles to the axis of centrifugation, whereas that of non-induced furrows (in control eggs centrifuged without pressure) is always parallel. Induced furrows tend to be displaced toward the light pole, quite drastically when higher pressures and forces are used.

4) Eggs treated during early prophase, just before the nuclear membrane disappears, require less pressure and force for breaking the nucleus and inducing furrows.

5) With eggs treated during late prophase, metaphase or anaphase, centrifugation alone, without pressure, suffices to induce furrowing.

Shortening of potassium depolarized muscle in different electric fields. Hidenobu Mashima and Arpad Csapo.

Muscles were marked off into several segments along their longitudinal axis by a fluorescent dye (Zn Cd S Phosphor), and were illuminated by ultraviolet light. A continuously moving film recorded the movement of these marks during shortening of the loaded muscle. Stimulation was made by electric fields set up in the perfusion Ringer, using 60 c/s a.c., rectified a.c., constant current and repeated square pulse d.c.

Then the muscle of the frog was rendered non-propagating by excess K, 16 mM/1. (substituted for Na). In the longitudinal a.c. field at 2 V/cm. the ends and the middle portion of the muscle contracted whereas the regions adjoining them did not. When relative shortening $\left(\frac{\text{non-propagating}}{\text{propagating}} \times 100\right)$ was plotted against the position of the segments along the length of the muscle, a W-shape curve was obtained. Increasing the field strength resulted in a gradual smoothening effect on the W-shaped curve as the segments close to the ends increasingly participated in the shortening resulting in a convex curve. At 8 V/cm. the convex curve was smooth. In d.c. fields only the cathodal end shortened.

When the longitudinal a.c. field was stepwise rotated so as to become eventually transverse, shortening along the length of the muscle became gradually more uniform and in our plot of relative shortening transverse stimulation yielded a straight horizontal line. In d.c. transverse field there was no shortening whatever.

Contraction at the ends of the non-propagating muscle in the longitudinal a.c. field or shortening of the whole muscle in the transverse a.c. field may be explained by the depolarizing effect of the applied current. But shortening in the middle portion, when the segments close to the ends are uncontracted, cannot be explained by the depolarizing action of the external longitudinal a.c. field.

Uterine strips from pregnant rabbits were rendered non-propagating by excess $K = 120$ mM/1.$\left(\frac{K_i}{K_o} = 1\right)$. Shortening was more excessive in the longitudinal than in the transverse a.c. field. In the longitudinal d.c. field the strip shortened along its whole length.

Cytophysiology of ultracentrifuged normal and neoplastic frog kidney cells.[1] G. M. Mateyko.

Viable cell populations of normal kidney cells and tumor cells (Lucké renal adenocarcinoma of *Rana pipiens*) were obtained by mechanical means or by trypsin treatment. To effect intracellular displacement and stratification of the subcellular components, the cells were subjected to high centrifugal fields (Spinco model L centrifuge). To prevent crushing, an isopycnotic cushion was established, the most satisfactory one being an isotonic, isosmotic stabilized colloidal silica with a density gradient maximum of 1.19. Most of the tumor cells had a density (*ca.* 1.123) greater than that of normal cells.

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[1] This investigation was supported in part by a research grant (C–3490) from the National Cancer Institute, U.S.P.H.S.

For the neoplastic population incipient stratification became evident, in a few cells, after centrifugation for 60 minutes at 60,000 G at 10° C., but was clearly defined only after ultracentrifugation at 110,000 G. A typical stratified cell was deformed into an ovoid shape with an agranular centripetal pole and a centrifugal end packed with small to large granules, with the nucleus shifted to the sub-equatorial or centrifugal zone. Normal cells showed an incipient stratification after two hours of ultracentrifugation at 110,000 G. Although they eventually stratified after four hours at 110,000 G, they never exhibited the range of subcellular displacement (centripetal lipid droplets, packing of granules into layers with sharply defined boundaries, pulled out and elongated nuclei, and extranuclear nucleoli) that was characteristic of malignant cells. Other cytophysical differences became exaggerated under high accelerations; for example, nucleolonemata remained undisplaced, but optically homogeneous (phase contrast observations) nucleoli exhibited a centrifugal shift within nuclei.

Relocation of the displaced components occurred imperceptibly in normal cells and rapidly in malignant cells. In the latter, within ten minutes after cessation of centrifugation, vigorous cytoplasmic turbulence was observed. The original cytoarchitectural configuration was generally restored within two hours. Ciliary movement in normal cells continued even after exposure to high centrifugal fields. Accordingly, cytoplasmic consistency and density differ in normal and malignant cells.

Cytochemical studies will complete the identification of stratified subcellular constituents, already partially determined by supravital dye techniques.

Studies on the accelerator cleavage factor recovered in homogenates of Arbacia punctulata ovaries. VALY MENKIN, LOUISE MENKIN AND RICHARD S. HEILMAN.[1]

The aqueous homogenate of the ovaries of Arbacia after centrifugation at 510 G for 10 minutes yields a supernatant fraction which in turn is centrifuged at 1150 G for 10 minutes. The supernatant (S_1), as a rule, contains predominantly the retarding factor. However, this fraction can be dialyzed for several days against distilled water in a refrigerator. The diffusate is found to contain the accelerator cleavage factor. Thus, by differential centrifugation followed by dialysis, the accelerator factor can be dissociated from any admixed retarding factor. Eventually the retarding factor will also tend to diffuse outward, but apparently this is a slower process, presumably owing to its larger molecular size. Following centrifugation to obtain the above described S_1 in the supernatant, the sediment in 0.25 M sucrose is centrifuged for 20 minutes at 8,170 G. The supernatant freed of mitochondrial particles is centrifuged one hour at 21,600 G in a Servall angle centrifuge. The soluble phase (S_2 of the accelerator factor) is then dialyzed against water in the cold for several days to eliminate some of the admixed retarding factor. In 9 out of 12 experiments, the accelerator factor has been recovered in the diffusate. In the S_1 diffusate, 6 out of 8 experiments revealed the presence of the accelerator factor, the average number of ova in the first blastomeric division being 44.5% in the experimental group and 22% in the controls. In the succeeding cleavage the averages were 46% and 26.5%, respectively. Absorption measurements of this active S_1 diffusate fraction by Dr. J. S. Roth have indicated a peak at 265 to 270 millimicra. Test for the presence of ATP by Dr. W. D. McElroy has been negative. The fraction is yellow in color, and in the visible range manifests a very tiny prominence at 390 millimicra. Studies, at the suggestion of Dr. O. Lindberg, by the phenol-water-ether extraction method indicate that the purified accelerator factor is a dinucleotide. Since uracil *per se* induces similar accelerating effects on cleavage, the possibility that the factor is a uracil dinucleotide is suggested.

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[1] Aided by grants from the Society of the Sigma Xi and the National Cancer Institute, U. S. Public Health Service.

Studies on the nature of the retarding cleavage factor in homogenates of sea urchin ovaries. VALY MENKIN, LOUISE MENKIN AND RICHARD S. HEILMAN.[1]

The aqueous homogenate of ovaries of *Arbacia punctulata* was purified by differential centrifugation as follows: centrifugation of the homogenate at 510 G for 10 minutes yielded a sediment, in turn treated with 0.25 *M* sucrose. The suspension was centrifuged at 21,600 G for 10 minutes. The supernatant was then centrifuged at the same speed for one hour. The resulting soluble phase (S_2), devoid of mitochondrial particles and probably of some of the microsomal fraction, was a clear yellow fluid which was very active in inducing a retardation in the cleavage development of ova. Usually the ova were exposed to the material for 20 minutes prior to fertilization. In two experiments the ova were first fertilized and then the S_2 fraction added. The results were similar in both procedures. From 0.5 to 1 ml. of the S_2 fraction were employed. It was found in 18 experiments, about 44 minutes following fertilization, that an average of 38.9% of control ova were in the first blastomeric division as compared with an average of only 2.9% in the experimental series. The retarding effect was likewise reflected in the second or further advanced cleavages, the average being 30.4% in the controls and 18.9% in the experimentals. The retarding factor was found to be heat-stable, withstanding boiling for 30 to 45 minutes. The active principle was diffusible through cellophane tubing of size 20/32. Absorption measurements by Dr. J. S. Roth and Mrs. Laura Inglis with a DU spectrophotometer indicated a peak at 265 to 270 millimicra. These facts suggest the nucleotide nature of the retarding factor. ATP and ADP were found present in one fraction but not in another active one by Dr. W. D. McElroy, by utilizing the firefly test. In the visible range there is also a peak at 400 millimicra. The nature of the yellow pigment is not yet clear. Addition of ascorbic acid induces temporary disappearance of the color, to be soon replaced by a deep orange color. This suggests that an oxidation-reduction system may also be present in the purified material.

Study of diatom populations on sand and mud flats in the Woods Hole area. E. T. MOUL AND DAVID MASON.

The presence of large populations of animals on and in mud flats of Barnstable harbor has raised the question of the source of primary production. Since few quantitative studies have been made of the diatom flora of mud flats, this study was undertaken.

Duplicate mud cores 2.5 cm. in diameter and 6 cm. deep were taken in plastic tubes from the mud flats of Barnstable harbor. The tubes were frozen and sectioned into layers. The samples from one tube were diluted with sea water and counts made of living diatoms. The duplicate sample was treated with acetone to extract chlorophyll. The number in the surface 2.5 cm. of mud varied from 9000 cells per cubic millimeter of mud in June to 500 cells per cubic millimeter in August. Living diatoms were found in the mud to a depth of 6 cm., decreasing markedly, however, below the surface layer. The number in the mud 5 to 6 cm. deep varied between 40 cells to 0 cells per cubic millimeter. The total calculated number of cells in a column of mud beneath a square meter of surface in one collection, for example, was 9.8×10^9 cells. These counts should be regarded as a relative index of abundance, since many of the diatoms grew tightly appressed to individual sand grains affording a possible source of error.

Chlorophyll was determined colorimetrically and presented in graph form along with the counts. There was general correspondence between the curves given by the two methods. In the example given above, the chlorophyll present in the column of mud was .948 gram per square meter. The order of magnitude of cells and chlorophyll present is in general agreement with that found for phytoplankton in a column of water beneath a square meter of sea surface in coastal waters.

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[1] Aided by grants from the Society of the Sigma Xi and the National Cancer Institute, U. S. Public Health Service.

The effect of varying concentrations of ribonucleic acid on the development of some marine embryos. M. C. NIU AND STEVEN D. DOUGLAS.

It has been demonstrated recently by the senior author that potency of isolated ribonucleic acid (RNA) from animal tissue is related to concentration. For *in vitro* differentiation of presumptive ectoderm, the concentration was 20–40 μg per milliliter in modified Holtfreter solution. A ten-fold concentration or dilution eliminated this effect upon differentiation. The present experiment was conducted to investigate the possible role of RNA concentrations in the development of some marine embryos.

Embryos of *Styela partita* (two-cell stage), obtained by artificial fertilization, were employed, as were those of *Arbacia punctulata*. They were grown in pasteurized sea water containing known amounts of RNA, isolated from the liver of *Raia erinacea*. Chemical tests for polysaccharide and protein in the RNA were negative.

At concentrations of 700 or more μg per milliliter, RNA arrested development at cleavage stage, 16–64 cells in Styela. At approximately 150–450 μg per milliliter rate of development was decreased; in this series, when the tadpoles hatched, the body became round and abnormal, a conspicuous otolith was present, and cross-sections revealed the reduction of neural tissue, particularly the cerebral vesicle. With concentration lowered from 100 to 5 μg per milliliter the rate of development increased, with a maximum, equal to or occasionally faster than the control. However, at concentrations lower than 3 μg per milliliter no appreciable effect was observed.

The effect of lower concentration is more striking in experiments with Arbacia embryos: approximately 4–16 μg per milliliter accelerated development. Thus, in a typical series, after treatment with RNA (4 μg per milliliter), for twenty-six hours, at room temperature (22° C.), most embryos attained the young prism to young pluteus stage, while in the control they were predominantly in the gastrula to prism stage.

In both forms the rate of development was influenced by RNA isolated from the liver of Raia. At optimal concentration of RNA, when the embryos hatched, neural structure in Styela appears to have been reduced.

Production of permanent lesions in living protoplasm. W. J. V. OSTERHOUT.

Colorless root cells of *Nitella* were used on account of their transparency.

Acid fuchsin did not enter the cell unless it was injured. Brilliant cresyl blue entered the vacuole more rapidly the higher the external pH value but as soon as the cell was injured the dye entered independently of the external pH values. These results were used as criteria of injury.

When a living cell was bent at the center, a lesion was formed which stained red with acid fuchsin but the rest of the cell remained colorless. When the cell was transferred to a solution containing no acid fuchsin, the dye escaped from the lesion and the cell lived for a long time in spite of the presence of the lesion.

The cyclosis stopped on bending but soon it was resumed and took a normal course unless the condition of the lesion prevented it.

In bending, cytoplasmic masses were often forced into the vacuole and they moved slowly and often revolved in the sap.

Sometimes the vacuole separated at the point of bending into two vacuoles but they later coalesced to form one vacuole again. If the cell was transferred to a solution of brilliant cresyl blue, the dye entered the vacuole more rapidly the higher the external pH value.

The vacuole stained red with neutral red separated into two red vacuoles at the point of bending but the space between them was colorless. Later the red vacuoles coalesced to form one red vacuole again.

A few cells freshly collected were found with some lesions. Their behavior toward acid fuchsin and brilliant cresyl blue was similar to that of the cell which had a lesion after bending.

These results indicate that permanent lesion can be present at one or more points on a cell without affecting the rest of the cell.

Selective permeability in relation to movement of water into living cells. W. J. V. Osterhout.

The purpose of the experiments was to determine if the movement of water controlled the rate of entrance of solutes into living cells.

A living cell was divided into two parts, A and B, by means of a vaseline seal. Water was placed at A and 0.025 M sucrose solution was placed at B. The water rapidly moved into the cell and travelled inside the cell from A to B, carrying with it particles which collected at B. No injury occurred.

When the water at A was replaced by a solution of acid fuchsin the water entered rapidly as before but the dye did not enter the cell unless it was injured. The acid fuchsin did not enter the living cell immersed in the dye solution unless it was injured. These experiments indicate that the movement of water into the cell does not control the rate of penetration of the solutes.

This was confirmed by experiments with basic dye, brilliant cresyl blue. If the water at A was replaced by brilliant cresyl blue at pH 5, in which the dye was chiefly in the form of ions, no dye entered the cell though the water entered rapidly as before. But the dye penetrated rapidly into the vacuole from a solution at pH 9 in which the dye was chiefly in the form of undissociated molecules, though the rate of movement of water remained unchanged. The dye was largely dissociated in the sap at pH 5.5. The dye moved from A to B and collected at B when the sucrose solution was at pH 9 but the dye escaped from the cell at B when the sucrose solution was at pH 5. When the cell was immersed in the dye solution the dye penetrated more rapidly at pH 9 and escaped from the cell more rapidly into the solution at pH 5 containing no dye.

Vital staining of eggs of Spisula solidissima by methylene blue.[1] Lionel I. Rebhun.

Washed eggs of *Spisula solidissima* are stained over a period of hours in solutions of methylene blue in sea water. Initial concentrations of dye used are one part in 1,000,000 and final ones, one part in 250,000. Small (½-micron) particles appear in the cytoplasm and become larger and darker with increased dye concentration and time of staining. After fertilization, these particles migrate to the asters. By the time the first polar body spindle is formed, the particles outline the asters, although many particles remain in other parts of the egg. By the first polar body stage, the particles are usually tightly organized about the asters and spindle. The particles may move rapidly (up to 2–3 microns per second) into the aster. They surround the female pronucleus when it is formed and then form a ring in the plane tangent to the pronuclei when these come into contact. The ring divides into two masses, each surrounding an aster, and each mass is distributed to one blastomere at cleavage. Usually the larger blastomere receives more particles. The particles gather at the peripheral cap of the re-formed blastomere nucleus and just before the next division this cap divides in two, each half surrounding an aster. After division, the peripheral cap forms again on each nucleus. The sequence of dividing into two before cleavage and cap reconstitution after cleavage is followed by the particles into late cleavage stages.

Electron micrographs of normal and stained eggs indicate that the dye causes the formation of vacuoles in the cytoplasm. These vacuoles appear to be swollen, distorted mitochondria. If so, only certain mitochondria appear susceptible and it is these which undergo the described changes in localization.

The biochemical basis for positive photokinesis of the starfish, Asterias forbesi.[2] Morris Rockstein and Melvin Rubenstein.

Pigments were extracted from the dorsal skin and "eyespots" of dark-adapted animals through acid buffer and into alkaline 2% digitonin solutions and their absorption spectra determined before and after exposure to light of wave-lengths from 300 to 700 mμ. Both types of extracts showed maximum absorbance at 495 mμ, with a primary minimum at 725 and a secondary minimum absorbance at 395 mμ. Exposure to sunlight, ultraviolet and visible light up to 450 mμ bleached either type of extract in a similar fashion, without altering visibly its natural violet color; maximum density loss occurred at 585 mμ, minimum loss occurred at 485 mμ; no change (isosbestic point) occurred at 520 mμ. Minimal changes in the entire absorption spectrum were observed for exposures at 500 and 550 mμ (*i.e.*, near the isosbestic point), whereas at 600 to 700 mμ, changes in absorption spectra for either kind of extract were exactly reciprocal of those obtained earlier at lower wave-lengths; *i.e.*, maximal density loss at 480 and minimal at 580 mμ, with the same isosbestic point of 520 mμ. The consistent occurrence of an isosbestic point at 520 mμ, as well as the similarity of their respective "difference spectra," indicates a common photosensitive biochemical component in the diffuse skin and more highly organized "eyespot" receptors of this animal. Their possible respective roles in the positive light orientation of this animal are being further explored in the form of correlative behavioral and pigment sensitivities to *different intensities* of different wave-lengths of light.

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The initiation and inhibition of cleavage of the Chaetopterus egg by ethyl urethane. HERBERT SCHUEL.

Because many of the same chemical and physical agents that initiate cell division also inhibit it, experiments were conducted for the past two summers on the effect of ethyl urethane on the cleavage of the Chaetopterus egg. Eggs placed in a 1% solution of urethane in sea water 5 minutes after insemination did not cleave, and viscosity studies made with the hand centrifuge indicated that the mitotic gelation, without which the spindle cannot form, did not occur. Similarly, fertilized eggs placed in 2% or 3% solutions did not cleave, and the mitotic gelation was again absent. Return of these eggs to normal sea water after the controls had cleaved showed the treatment with a 3% solution to be irreversible while the treatment with the 2% solution was only partly reversible. Almost all the eggs treated with a 1% solution cleaved within 15 minutes after being returned to sea water; a rapid and sharp increase in viscosity culminated in cleavage.

Unfertilized eggs exposed to a 3% solution for periods ranging from 10 to 60 minutes began to cleave and develop, with a peak of activity evident at about 30 minutes exposure. Optimum cleavages ranged from 20% to 88%. Viscosity studies showed an increase of the same order of magnitude as the mitotic gelation. A 2% solution would only occasionally initiate cleavage and development, indicating it to be a threshold concentration.

Larvae produced either from unfertilized eggs treated with 2% or 3% solutions or from fertilized eggs treated with 1% or 2% solutions appeared to be morphologically abnormal, and, instead of swimming about, rotated rapidly in place on the bottom of the dish.

Preliminary studies of the ontogeny of schooling behavior in the silversides, Menidia menidia. EVELYN SHAW.

The development of schooling was studied to try to clarify some of the factors involved in the interaction of fish to fish when the first school is formed. It was found that schooling does not appear immediately after hatching but develops gradually as the fish matures. Sixty five individual fish, swimming freely in glass bowls, were observed in their responses to other fish. No schooling occurred in fish younger than 9 days. Between 10 and 15 days of age, schooling was brief and sporadic after a long period of no response. On the 17th day all of the fish responded instantly and schooling continued throughout the observation. At this time the fry had grown from 5 mm. at hatching to 12 mm., the size of the smallest schooling fishes found in the field and in our laboratory aquarium.

The initial stimulus to schooling appears to be a visual one. This was tested by placing a fish in a sealed glass tube with a fish who was swimming freely in a bowl. Fifteen out of twenty fish attempted to line up with the fish in the tube. After one or two minutes schooling ceased and the fish vibrated near the tube, suggesting that additional stimuli may be necessary to the continuance of schooling.

In another series of experiments eighteen fish were deprived of early social contact with species mates to see if this environmental condition would influence the appearance of schooling. On the 17th day of development species mates were introduced into their bowls. Nine of the separated fish schooled within the first minute while the remaining nine did not school during the entire twenty minute observation. After twenty four hours, however, schooling was noted in all of the test fish. Evidently lack of contact with species mates does not alter the pattern of schooling, but initially inhibits the number of fish participating in this behavior.

This work was supported by an ONR contract with the MBL.

Effects of x-ray irradiation on two strains of Tetrahymena corlissi.[1] CARL CASKEY SPEIDEL.

Two strains of a recently named new species, *Tetrahymena corlissi,* have been subjected to severe successive x-ray treatments. Strain "W" was found by Speidel at Woods Hole, Massachusetts, as a facultative parasite in tadpoles of *Bufo.* Strain "C" was found by Thompson at Charlottesville, Virginia, in a moribund larva of *Pseudotriton.* Structurally and functionally the two strains seemed identical. A micronucleus was present. The tetrahymenae reproduced rapidly by binary fission and by reproductive cysts. Conjugation was not seen. During the summer of 1956 strain C was subjected to a series of 9 x-ray treatments over a period of 60 days. The separate doses ranged in strength from 400–600 kr; the cumulative dose totaled 4217 kr. During the summer of 1957 strain W was subjected to a similar series of 9 x-ray treatments over a period of 57 days. The cumulative dose for this strain totaled 4500 kr. Surviving tetrahymenae in each strain gave rise to progeny containing many amicronucleate individuals. Clones of amicronucleate tetrahymenae were readily established from both x-rayed strains. During the summer of 1957 after a 10-month interval of recovery, cultures of amicronucleate tetrahymenae derived from strain C were subjected to 5 additional x-ray treatments totaling 2900 kr over a period of 41 days. The separate doses ranged in strength from 500–700 kr. The total cumulative dose for both summers received by this strain amounted to 7017 kr. The surviving progeny exhibited no marked difference in radiosensitivity or radioresistance as compared with normal tetrahymenae. There was no conspicuous lasting effect on the capacity and speed of reproduction.

Enzymatic dissociation of sponge cells.[2] MELVIN SPIEGEL AND CARROLL METCALF.

The effect of several enzymes on sponge reaggregation was studied in an effort to prepare completely dissociated cell suspensions. The following enzyme solutions in artificial sea water (Tyler) were used: 2% crude protease, pH 7.6; 0.2% crystalline trypsin, pH 7.7; 0.2% crystalline chymotrypsin, pH 7.7; 2% crude papain containing 100 mgm% cysteine, pH 7.0; 2% steapsin, pH 7.0. The stomach juices of the green crab, *Carcinus maenas,* and of the blue crab, *Callinectes sapidus,* were also used. Two-tenths ml. of the expressate obtained by pressing 2 gm. of the sponge *Microciona prolifera* through bolting cloth into 20 ml. of artificial sea water was then added to 3 ml. of each enzyme solution. Three ml. of artificial sea water served as control.

Trypsin, protease, chymotrypsin, and steapsin had no effect on dissociation. Aggregates formed, rounded up within 6–8 hours, and adhered to glass but did not exhibit the flattening and spreading out which normally occur 24 hours after dispersal. In papain, aggregates were formed which failed to round up with the cells loosely adhering to one another. Attempts to obtain complete dissociation by passing the papain-suspension through a fine pipette led to cytolysis.

In stomach juice of the green crab, at first large aggregates were formed but the peripheral cells were in loose contact and 18 hours after dispersal many small aggregates were formed. No rounding up occurred. In blue crab stomach juice little reaggregation took place. In the center of the largest aggregrates which were formed condensations of closely packed cells were noted with loosely connected cells between these condensations. Eighteen hours after dispersal a good cell suspension could be obtained by passage through a pipette.

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[1] This investigation was supported by a research grant (PHS RG-4326 R) from the National Institutes of Health, Public Health Service.

[2] Supported by Research Grant E-1365 from the National Institute of Allergy and Infectious Diseases.

Viability of dissociated frozen-thawed sponge cells.[1] MELVIN SPIEGEL AND CARROLL METCALF.

A cell suspension of the sponge *Microciona prolifera* was prepared by pressing 2 gm. of sponge through bolting cloth into 20 ml. of filtered sea water. Two-ml. aliquots were transferred to 8 ml. of varying concentrations of ethylene glycol or glycerine in filtered sea water, frozen at − 12° C.; after 1–24 hours rapidly thawed at 18° C. and washed 3 times with filtered sea water. A control suspension in filtered sea water was treated in identical fashion. Cells frozen in ethylene glycol did not survive the treatment. After freezing in 10% glycerine, approximately 30% free cells were noted but the amoebocytes exhibited no pseudopodial movement and no collar cells with a beating flagellum were noted. No reaggregation of cells occurred. After freezing in 20% and 30% glycerine, only 10% free cells were noted with no cell movement. In 40% glycerine the amoebocytes showed pseudopodial movement and a few collar cells with beating flagellum were noted. Twenty-four hours later the normal rounding up of aggregates had not occurred with no aggregate adherence to glass. Dialysis vs. filtered sea water did not improve survival. Further reaggregation did not occur after culture for 7 days. Similar results are obtained with unfrozen filtered sea water-glycerine suspensions. After thawing of filtered sea water suspensions the amoebocytes exhibit normal psudopodial movement and adhere to glass. No collar cell movement was noted. Normal reaggregation occurs during the first 4 hours after thawing but within 8 hours cytolysis sets in and reaggregation ceases. Varying the time for freezing and for thawing has not furthered survival. The results show that sponge cells can survive freezing and thawing in glycerine-filtered sea water solutions and in sea water alone. Neither glycerine nor ethylene glycol offers any protective advantage compared to filtered sea water.

The reaggregation of Microciona cells in culture media.[1] MELVIN SPIEGEL AND CARROLL METCALF.

Although aggregates of sponge cells which have been dissociated by passage through bolting cloth into sea water can easily regenerate to form a new sponge when placed in a live-car, attempts to rear such aggregates in culture media, synthetic or otherwise, have failed. In an effort to circumvent this difficulty the following culture media were used: A. l-leucine, 31 mgm.; dl-phenylalanine, 10 mgm.; dl-tryptophane, 8 mgm.; l-cysteine, 2 mgm.; l-arginine, 16 mgm.; dl-methionine, 26 mgm.; glucose, 1.7 gm.; 200 ml. artificial sea water (Tyler). B. 1%, 0.5%, and 0.1% glucose in artificial sea water. C. 1%, 0.5%, and 0.1% egg albumen in artificial sea water. D. Medium A minus glucose. E. 1%, 0.5%, and 0.1% galactose in artificial sea water. F. 1%, 0.5%, and 0.1% levulose in artificial sea water. G. 0.5% boiled dried yeast in artificial sea water. All solutions were adjusted to pH 8.2. Filtered sea water and artificial sea water served as controls. Two ml. of a cell suspension, obtained by pressing 2 gm. of *Microciona prolifera* through bolting cloth in 20 ml. of artificial sea water, were added to 8 ml. of each medium.

In media A-F reaggregation was impeded. More numerous but smaller aggregates were formed than in the controls. These did not regenerate further. In medium G a functional sponge was formed with canals and chambers lined with beating collar cells and raised oscula. When yeast cells stained with neutral red were added to this sponge within two hours the entire regenerate was stained. In control media canals and chambers were formed but neither water currents nor oscula were observed.

Uptake of amino acids from sea water by ciliary-mucoid filter feeding animals. G. C. STEPHENS AND R. A. SCHINSKE.

Several reports in the literature concerning filter feeding animals indicate that some may retain small quantities of dissolved protein. The following observations were undertaken to

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[1] Supported by Research Grant E-1365 from the National Institute of Allergy and Infectious Diseases.

determine whether ciliary-mucoid filter feeders are capable of taking up small organic molecules. An initial set of observations was made with 2 mM. glycine (150 mg./liter sea water) using the slipper limpet, *Crepidula fornicata;* the mussel, *Mytilus edulis;* and the coral, *Astrangia danae.* Single mussels, or several Crepidula, or an Astrangia colony, were placed in approximately 250 ml. of solution. This solution was sampled periodically and the amino acid concentration measured colorimetrically using a ninhydrin reaction. Control samples were run in parallel, and colorimetric determinations were done in triplicate. Considerable variation in rate of uptake was observed, particularly in the case of Mytilus and Crepidula. However, the following results may be cited as typical. Mytilus in one experiment removed 10% of the glycine in five hours and 45% in twenty-four hours, while Crepidula removed 15% and 65% and Astrangia, 20% and 97%.

Astrangia was chosen as a suitable animal for additional experiments. Five readily soluble amino acids were selected to cover a range of isoelectric points. These were glutamic acid, methionine, glycine, alanine and arginine whose isoelectric points are, respectively, 3.08, 5.7, 6.06, 6.1 and 10.76. Uptake in all cases was roughly comparable to that for glycine. These observations were made by sampling at intervals about one liter of the solution in which several colonies had been immersed. Various controls included rocks from which the colonies had been removed but which still included masses of the sponge, Cliona, and rocks together with the crushed coral organisms. These did not show significant uptake.

Observations were also made on the rate of removal in concentrations from 0.4 mM. to 10.0 mM. No striking difference in rate of uptake was apparent over this 25-fold concentration difference.

The effect of 2,4-dichlorophenoxyacetic acid on oxygen consumption in Uca pugnax. FREDERICK N. SUDAK AND C. LLOYD CLAFF.

The lethal concentration of 2,4-dichlorophenoxyacetic acid, as determined by injection directly into the hemocoel, was found to be 400 mg./kg. wet body weight. The L.D. 50/24 hrs. was determined as 325 mg./kg. wet body weight. Symptoms resembling myotonia, found in homeothermic vertebrates treated with this compound, were produced with concentrations between 200 and 275 mg./kg. wet body weight. Muscular movements were initiated after some delay but once movement was started, the animal continued to move without any apparent difficulty.

Oxygen consumption of crabs treated with 2,4-D was measured at 17.5° C. using a modified Scholander respirometer for aquatic animals. A decrease in O_2 consumption of an average of 78% occurred within 60 minutes after injection of 200 to 250 mg./kg. into the ventral hemocoel. Oxygen consumption returned to pre-injection levels 10–15 hours later. Control animals, in simultaneous experiments, were injected with equal volumes of filtered sea water. O_2 consumption was increased for 30 minutes after injections followed by a recovery to pre-injection levels.

Metabolic responses of albino rats treated with 2,4-dichlorophenoxyacetic acid to changes in ambient temperature. FREDERICK N. SUDAK, C. LLOYD CLAFF AND MARVIN H. CANTOR.

Twelve hour fasted male albino rats injected with 300 mg./kg. of 2,4-dichlorophenoxyacetic acid increased their CO_2 output 43% with no change in body temperature when kept in an isothermic environment of 30° C. for 40 to 60 minutes after injection. Carbon dioxide production increased 85% accompanied by a rise of 2° C. in body temperature when the ambient temperature was increased to 33° C. Animals treated with this compound died within 20 minutes in an environmental temperature of 35° C. Rectal temperatures taken at the time of death were above 40° C. Control animals injected with an equal volume of physiological saline decreased their CO_2 production 14% at both isothermic and 33° C. temperatures while rectal temperatures remained constant. Carbon dioxide production returned to the control levels taken at room temperature and the rectal temperatures increased 1° C. when the ambient temperature was increased to 35° C. Both groups of animals perspired freely at this temperature.

Myotonia induced in albino rats with subcutaneous injections with 2,4-dichlorophenoxyacetic acid persists for 18 to 24 hours after injection but the duration of the metabolic response to increased ambient temperature is much shorter, *i.e.*, seven hours. Animals injected two hours earlier died within 20 minutes when the ambient temperature was increased from 30° C. to 35° C. The rectal temperature of these animals increased from 37° C. to above 40° C. Animals injected five hours earlier survived the 20-minute exposure to 35° C. heat. CO_2 production increased 40% and rectal temperature rose 2° C. Metabolic and body temperature responses were the same as those of control animals when the ambient temperature was raised to 35° C. seven hours after an injection of 2,4 D.

Metabolic activity of rats treated with 2,4 D behaved much like that of a poikilothermic animal for seven hours after injection. The calculated Q_{10} of these animals was 3.5 in heat and 1.0 when they were placed in a cold environment.

Expansion of the pre-placental yolk-sac in Mustelus canis. LOIS E. TEWINKEL.

Yolk-sac folds and villi were mentioned by ten Cate-Hoedemaker and Ranzi in detailed accounts of the placenta of *Mustelus laevis,* but the extent of yolk-sac growth was not emphasized, undoubtedly because they had no late pre-placental stages. Mahadevan, in several Indian Selachii (Scoliodon), noted elaborate yolk-sac growth, but made no measurements.

Scarcity of pregnant smooth dogfish at Woods Hole has prevented a thorough-going study, but, when yolk-sacs of 40 mm. embryos are compared with those of 55, 65, and 109 mm. embryos, it is clear that in *Mustelus canis* the yolk-sac grows enormously as yolk is depleted. Spreads of yolk-sacs, dried and measured on millimeter paper, show an increase from an initial area of 1260 $mm.^2$ for a 40 mm. embryo (where the yolk-sac has not begun expansion) to 4474 $mm.^2$ for a 65 mm. embryo, and over 10,000 $mm.^2$ for a 109 mm. specimen.

Although yolk is still present in all sacs and in vitelline ducts of embryos 50 mm. and longer in the stages studied, progressive growth in surface area of the sac as yolk decreases, suggests that, in addition to yolk absorption, material is being taken in from fluid diffusing into the egg-case cavity from uterine sources. That absorption of water occurs in these pre-placental stages is shown in a comparison of wet and dry weights of embryos plus yolk-sacs when embryos measured approximately 17, 34, and 60 mm. The following figures for each class represent an average of three specimens. Wet weights were: 2.10, 2.722, and 4.22 grams, respectively; and dry weights: 1.016, 1.19, and 1.395 grams. Thus, in a 60 mm. embryo and yolk-sac, water content is 2.6 times that in a 17 mm. specimen and has risen to 67% of the wet weight as compared with 52%. Ash determinations have not yet been made.

The hexose monophosphate shunt in marine invertebrates. CLAUDE A. VILLEE, JANET LORING AND FREDERICA WELLINGTON.

The hexose monophosphate shunt provides a path for the metabolism of glucose which is an alternative to the Embden-Meyerhof glycolytic cycle. Glucose-6-phosphate is oxidized to 6-phosphogluconic acid which is then oxidatively cleaved to CO_2 (from carbon 1) and the pentose, ribulose-5-phosphate. An estimate of the fraction of glucose metabolized by each path is obtained by comparing the rates of metabolism of glucose-6-C^{14} and glucose-1-C^{14} to $C^{14}O_2$ (G_6/G_1). The rate of carbon 1 of glucose to CO_2 is taken as the sum of the two paths and that of carbon 6 is taken as the glycolytic path alone. Krahl had shown that the monophosphate shunt is an important path in the metabolism of developing *Arbacia* eggs and that it could account for essentially all of the oxygen consumed. We have confirmed his results and extended the study to a variety of excised tissues from other echinoderms, annelids and molluscs. In our experiments the G_6/G_1 ratio (mean of 12 experiments) for unfertilized *Arbacia* eggs was 0.07, for fertilized eggs (12 hours after fertilization) was 0.20 and for sperm was 0.23. This confirms Krahl's finding that the glycolytic path becomes more important as development proceeds. From the G_6/G_1 ratio it appears that the hexose monophosphate shunt is an important metabolic pathway in *Busycon* red retractor muscle and digestive gland, in *Thyone* respiratory tree and gut, and in *Chaetopterus* and *Arenicola* muscle and gonads. In marked contrast, the G_6/G_1 ratio was unity in *Pecten* gill and mantle and in *Loligo* gill. The gills of these molluscs have high rates of oxygen consumption but it would appear that all of the glucose is metabolized via a glycolytic system.

X-irradiation of the giant multinucleate ameba, Chaos chaos. RALPH WICHTERMAN.

Specimens from the original 1936 Schaeffer strain were irradiated singly with doses from 20,000 *r* to 80,000 *r* in steps of 10,000 *r* and with 120,000 *r* — the LD 50, 72 hours. A single ameba was placed in either a one- or two-cc. closed Lucite chamber free of air and containing only 10 parts of spring and 90 parts of glass-distilled water. Five or 10 of the isolated amebas were irradiated at one time. Immediately after irradiation, the water was replaced and *Paramecium multimicronucleatum* and *Chilomonas* added as food. The amebas were maintained in culture until either their death or until successful mass clonal cultures were established. When an irradiated ameba produced a clone of at least 30 specimens, it was considered a successful mass culture. Occasionally some of the clones were allowed to produce several hundred amebas.

This multinucleate organism may divide commonly into two or three, occasionally more, daughters. As a more reliable measure, all progeny of a divided specimen were kept in the same container and totalled since it was found that some early daughters of an irradiated ameba may die before dividing later, while others may survive to divide again and produce successful mass clonal cultures.

As has been observed for a number of other Protozoa, the greater the dosage, the greater the delay in the first division following irradiation. Thereafter—if the cell is to divide again—the time between the next and succeeding divisions is progressively shorter. There is no consistency in the number of daughters produced by either a single unirradiated control specimen or an irradiated one when observed daily over a period of at least 2 weeks.

After irradiation with 40,000 *r*, some isolated specimens produced as many as 10 or less amebas which died while others yielded clones of hundreds. On the other hand, a similarly irradiated specimen may live for at least 23 days without dividing.

With 50,000 *r* and 60,000 *r*, it may require as long as 10–17 days before the ameba first divides to eventually yield a mass clonal culture. Generally a cell which divides earlier than others similarly irradiated is one that is more likely to produce a mass culture.

Successful mass cultures have been established after the irradiation of single specimens with from 20,000 *r* to 70,000 *r*.

Some physiological characteristics of the fish heart. CHARLES G. WILBER.

In mammals it is well known that the average heart-rate varies inversely with body size. In the present work fish, which varied in size from pipefish to striped bass, were studied electrographically to ascertain whether a similar relation was true. It was found that the average resting heart-rate in fish varies inversely with body size and an equation expressing the relationship has been derived. Teleosts fit the plotted curve very well; elasmobranchs do not, although they apparently fit a special curve for themselves. Various drugs were tested with the intact hearts of different species of fish. Fundulus heart responds readily to atropine with an increase of rate. Darstine has a similar but more pronounced effect. Atropine is not very effective in the toadfish. However, darstine in relatively large doses brings about complete A-V dissociation; an idioventricular rhythm is established; atrial rate is slightly depressed. The interpretation of the results is not easy. Preliminary results have been obtained which indicate that in the sea robin an increase in temperature of the fish brings about an increase of blood pressure as measured directly from the first gill vessel. The heart rate in the intact fish also increases with temperature but in our work a temperature coefficient of 2 or 3 has not been routinely found. This is in accord with our data for alligators. These studies are supported by the National Science Foundation.

LALOR FELLOWSHIP REPORTS

Accessory fiber synaptic excitation of squid stellar giant axons. S. H. BRYANT.

In the excised squid (*Loligo pealii*) stellate ganglion preparation equilibrated in oxygen-saturated medium, it is possible to observe excitatory behavior of the proximal (accessory) synapses of the stellar giant axons.

The large pre-ganglionic axon to the giant (distal) synapses was removed from the nerve trunk near the ganglion for stimulation. The accessory fiber and other smaller giant axons were stimulated in the nerve trunk 2 to 3 cm. from the ganglion. Selective stimulation of the remaining pre-ganglionic axons indicated that the proximal synapses were excited mainly by the accessory fiber (Young), occasionally by one or more of the small giants, but not by the small axons. There was no evidence of giant synapse inhibition by stimulation of the pre-ganglionic giant axons.

The proximal synapses are more sensitive to oxygen lack and can be fatigued independently of, and earlier than, the giant synapses. Intracellular recording from the last stellar axon near its inflection in the ganglion reveals both the proximal and giant synapse excitatory post synaptic potentials (e.p.s.p.). The proximal e.p.s.p. was preceded by a synaptic delay and had a temporal form similar to that of the giant synapse. Depolarization levels for spike initiation were nearly the same in spite of different initial slopes.

When the e.p.s.p. of the proximal synapses is timed to arrive shortly after the giant synapse is excited, it can abolish the undershoot and add to the middle of the falling phase of the spike. If it arrives about 1 to 2 msec. later, it can give rise to a second spike, depending upon the refractory period of the post axon. Parallel results were obtained when the proximal e.p.s.p. arrived before that of the giant synapse.

In preparations where both sets of synapses were critically fatigued it was possible to get addition of excitation when the e.p.s.p.'s were added in their early rising phase. If they were added later there was addition of depolarization but not necessarily excitation.

The site of origin of the nerve impulse in the lobster stretch receptor. J. F. Case, C. Edwards,[1] R. Gesteland and D. Ottoson.

The cell body and axon of the lobster stretch receptor organ are clearly visible with dark field illumination. Thus, microelectrodes may be placed on visually well defined sites to record potential changes when the cell is activated by stretch or by antidromic stimulation. Simultaneous recordings have been made with two glass micro-electrodes of potential changes in the cell body and at various places along the axon of the lateral seventh thoracic stretch receptor immersed in saline. An analysis of latencies and potential configurations was made to locate the site of origin of the conducted impulses.

In a lightly stretched receptor the potential change of the soma starts with a positivity followed by the negative spike. In the axon the impulse lacks the initial positive phase if recorded from a point near the cell body. The start of the positive phase of the cell body response is synchronous with the start of the negative phase of the potential change in the axon; the cell body responds with an impulse 0.1–0.3 msec. later than the initial part of the axon. If antidromic spikes are recorded with the electrodes at the same position, the potential changes in the axon start with a positive phase while the cell body response is identical to that set up by stretch. The soma impulse follows the axonal spike by about the same interval whether set up by stretch or by antidromic stimulation. On the basis of these observations the conclusion is drawn that the impulse set up by stretch starts in the axon near to the cell body and then propagates out along the axon as well as back into the cell body.

Phosphoarginine and arginine phosphokinase from Homarus americanus. L. Lorand.

Enzyme systems capable of producing adenosinetriphosphate from adenosinediphosphate seem to be involved in the relaxation of muscle, as suggested by previous studies with creatine phosphate and phosphoenolpyruvate. It would be of interest to test the effect of an invertebrate phosphate donor system, such as phosphoarginine, on vertebrate muscle. As a preliminary to these studies, phosphoarginine and the enzyme arginine phosphokinase had to be prepared.

Since phosphoarginine cannot be synthesized, its isolation from *Homarus americanus* was attempted. The tail muscle was homogenized without prior freezing in liquid nitrogen, otherwise the procedure of Ennor, Morrison and Rosenberg (Biochemical Journal, 1956), given for

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[1] Fellow of the Lalor Foundation.

Jasus lalandii, was followed. Phosphoarginine could be obtained from the American lobster, although in considerably lower yield than from the Australian species.

The enzyme arginine phosphokinase was also purified from *Homarus americanus* by the method given for *Jasus verrauxi* by Morrison, Griffiths and Ennor (Biochemical Journal, 1957).

The effect of the invertebrate phosphate donor system on the relaxation process is now being studied.

This work was carried out during the tenure of a Lalor Fellowship.

Incorporation of labeled iron into hemerythrin. MARTIN P. SCHULMAN.

Hemerythrin is an iron-containing respiratory pigment that occurs in nucleated coelomic corpuscles of *Phascolosoma gouldii*. It combines reversibly with O_2, is not an iron-porphyrin compound, and can be crystallized readily (Florkin, 1932). When coelomic cells or hemolysates of these cells were incubated with $Fe^{59}Cl_3$, the isolated oxyhemerythrin contained Fe^{59}. The incorporation of Fe^{59} into oxyhemerythrin in a hemolysate was fifteen times greater than in an intact cell preparation, suggesting low permeability of these cells to ferric iron. Although the incorporation of Fe^{59} into hemerythrin in hemolysates was not linear past one hour incubation, the uptake of Fe^{59} after five hours was still considerable. Experiments that ruled out a non-enzymatic exchange of Fe^{59} with hemerythrin were: (1) at 0° intact cells and hemolysates did not incorporate Fe^{59} into hemerythrin; (2) incubating $Fe^{59}Cl_3$ for varying lengths of time with oxyhemerythrin or hemerythrin reduced by deoxygenation did not result in any uptake of Fe^{59} by the pigment. Oxyhemerythrin was crystallized four times to rid the pigment of ionic Fe^{59}; at this stage the specific activity of oxyhemerythrin that contained Fe^{59} as a result of incubation was constant. Oxyhemerythrin containing Fe^{59} was assayed by the following procedure: a known volume of pigment in 0.35 M NaCl was assayed for radioactivity in a scintillation well counter, while another aliquot was measured spectrophotometrically to determine the concentration of hemerythrin. The purity of each sample was determined by measuring the optical densities at 280 mμ, 330mμ and 500 mμ and calculating the following ratios: 280/330 and 330/500 which were 5.27 and 3.16 ± 1%, respectively, for the best preparations. The specific activity was expressed as counts per minute per optical density unit at 330 mμ. It is believed that the incorporation of Fe^{59} into hemerythrin is enzymatic and represents a biosynthesis of the molecule.

Aided by AEC contract AT(30-1)1343 at Marine Biological Laboratory, Woods Hole, Mass.

Heme synthesis in peripheral blood of marine fishes. MARTIN P. SCHULMAN AND GEORGE A. LAMB.

Twenty-five per cent suspensions of twice-washed erythrocytes in saline containing $Fe^{59}Cl_3$ were shaken in air for two hours at 22° C. The cells were then washed with saline, hemoglobin carrier added, and hemin isolated and purified by Fisher's procedure. Red cells were stained supravitally with brilliant cresyl blue according to Dawson (1933), who found that bloods of different fishes varied in their percentages of immature cells. Our study showed that the incorporation of Fe^{59} into heme varied with the degree of reticulation in each blood. For example, the red cells of the toadfish (*Opsanus tau*) had less than 0.5% immature forms and incorporated negligible Fe^{59} into heme. Other teleosts, such as the common sea robin (*Prionotus carolinus*), scup (*Stenotomus chrysops*), king mackerel (*Scomberomorus regalis*) and bonito (*Sarda sarda*), had 5 to 10% immature red cells and incorporated Fe^{59} in proportion to the degree of reticulation. In the elasmobranchs studied—smooth dogfish (*Mustelus canis*), spiny dogfish (*Squalus acanthias*), spotted skate (*Raja diaphanes*), dusty shark (*Carcharhinus obscurus*)— 25 to 30% of the red cells were immature and the uptake of Fe^{59} was considerable. Since the bloods of the elasmobranchs contained about four times as many immature cells as did the teleost bloods, it was expected that the incorporation of Fe^{59} would also be increased fourfold. However, the incorporation was much greater and may be explained by the increased density of reticulum per immature cell of the elasmobranchs. Additional studies on red cells of Mustelus showed that Fe^{59} incorporation was linear during the incubation, addition of glycine and succinate, precursors of the protoporphyrin moiety of heme, did not stimulate the uptake of Fe^{59}, and Fe^{59} incorporation into heme was markedly lowered when cells were incubated in serum.

Aided by USPHS grant C-1852 and AEC contract AT(30-1) 1343 at Marine Biological Laboratory, Woods Hole, Mass.

The effects of metabolic inhibitors on ion distribution and membrane potential in muscle fibers of the green crab, Carcinides maenas. WILLIAM K. STEPHENSON.

Fiber ion compositions were determined in muscle fibers from the flexor in the meropodite of the green crab, *Carcinides maenas.* Sodium and potassium analyses were carried out by flame photometry, and chloride was measured with a mercurimetric titration. Fresh muscle has an average fiber potassium concentration of 156 mEq/liter fiber water (lfw) (17 determinations), a sodium concentration of 72 mEq/lfw (17 determinations), and a chloride concentration of 94 mEq/lfw (13 determinations). In calculating fiber ion concentrations an extracellular space value of 5% (of wet weight), as determined by the inulin method, and a dry weight of 22% were used. Microelectrode determinations of membrane potentials on fresh fibers bathed in blood gave an average value of 58 mV for 43 fibers from 5 crabs. Upon exposure to natural sea water or to MBL-formula sea water the potential rises to an average of 69 mV over a period of 30–60 minutes.

If MBL sea water is perfused through the removed, but unopened, leg segments, the fiber potassium falls less than 5% over a 6-hour period. 5 mM iodoacetic acid (IAA), 5 mM cyanide (CN), and 0.2 mM dinitrophenol (DNP), added to MBL sea water, were similarly perfused through the preparation and their effects on the ion balance were observed. In terms of increasing the loss of potassium the effects of the inhibitors were in the following order: $\mathrm{CN} < \mathrm{IAA} < \mathrm{DNP} \cong \mathrm{DNP} + \mathrm{CN} \cong \mathrm{IAA} + \mathrm{CN} < \mathrm{DNP} + \mathrm{IAA}$. The effects of inhibition on sodium and chloride concentrations were too equivocal to include here. The inhibitors also produced a decrease of membrane potential in the following order: $\mathrm{CN} < \mathrm{DNP} \cong \mathrm{DNP} + \mathrm{CN} < \mathrm{IAA} < \mathrm{DNP} + \mathrm{IAA}$ (the effect of CN IAA was not clear). IAA thus produces a rapid effect on membrane potential without markedly altering the potassium, sodium, or chloride distributions.

In vitro studies on intestinal absorption of fish. T. HASTINGS WILSON.

The ability of fish intestine to transport sugars and amino acids across the wall against a concentration gradient was tested with an *in vitro* technique. A tied sac of everted small intestine was filled with saline (usually 2 ml.), placed in a 50-ml. Erlenmeyer flask containing 3 ml. of saline and gassed with 100% oxygen. The saline composition was as follows: NaCl (0.21 *M*), $MgSO_4$ (0.002 *M*), $NaHCO_3$ (0.002 *M*), K_2HPO_4; pH 7.0 (0.01 *M*).

Proline and glycine were actively transported by the intestine of the puffer (*Spheroides maculatus*). In a representative experiment L-proline (50 mg%) was added to the saline on each side of the intestinal wall. At the end of one hour of incubation at 26° the concentration of proline on the mucosal side fell to 24 mg% while that on the serosal side rose to 65 mg%. There was a net loss of proline from the system which could be partially accounted for by the appearance on the mucosal side of an amino acid chromatographically similar to glutamic acid.

In contrast to the results obtained with amino acids, glucose was not transported across the intestinal wall against a gradient by *in vitro* preparations from any of the following fish: sea robin (*Prionotus carolinus*), scup (*Stenotomus chrysops*), toadfish (*Opsanus tau*) or puffer. Galactose and 6-deoxy-D-glucose were also tested with the puffer gut preparation and were not transported.

During the *in vitro* incubation the mucosal side of puffer and sea robin intestine became progressively alkaline (from pH 7.0 to about 7.6). This alkaline intestinal secretion was confirmed *in vivo* in the case of the sea robin.

Phylogenesis of plasma proteins and plasma cells. I. Starch gel zone electrophoresis of sera from marine invertebrates and fishes. KENNETH R. WOODS AND RALPH L. ENGLE, JR.

The method of zone electrophoresis in starch gel described by O. Smithies in 1955 has been used to separate proteins from the sera of several marine invertebrates, cartilagenous and bony fishes, and a few amphibians, reptiles, and lower mammals. From six to twelve specimens of each form were analyzed. The resulting serum electrophoretograms exhibited a high degree of intraspecies reproducibility and a high degree of interspecies specificity.

Closely related decapod crustacea sometimes gave similar serum electrophoretograms, but exceptions were found. More than one major protein fraction was always obtained from hemocyanin-containing sera. This finding was attributed to the dissociation of hemocyanin into non-identical components at the high pH (9.0) of the starch gels. None of the invertebrates examined yielded serum proteins with the electrophoretic properties of human gamma-globulin.

Each of seven species of the elasmobranchs produced its own unique serum electrophoretogram. These sera contained fractions which migrated into the cathodic region of the gel in a fashion similar to the movement of human gamma-globulin. Bony fishes gave highly specific patterns, but proteins corresponding electrophoretically to human gamma-globulins were either greatly reduced or absent. Among the bony fishes the occurrence of a protein corresponding to albumin and of another with a slightly slower mobility than beta globulin were consistently observed. Other proteins of random mobilities and distributions were present, giving each bony fish species a characteristic pattern.

Despite the few exceptions which might prove to have important biological significance, these studies indicate that serum electrophoretic patterns of closely related species have a remarkable degree of specificity. These findings suggest that starch gel electrophoresis of serum proteins may contribute valuable information to population studies, comparative immunology, taxonomic problems, and to other considerations of biochemical individuality.

Phylogenesis of plasma proteins and plasma cells. II. Observations on the occurrence of plasma cells in marine invertebrates and fishes. RALPH L. ENGLE, JR. AND KENNETH R. WOODS.

Microscopic examinations were conducted to determine whether or not plasma cells are present in invertebrates and cold-blooded vertebrates. Plasma cells in mammals are recognized by their eccentric nucleus and basophilic granuloplasm usually containing a paranuclear clear zone. Cells in lower forms having the same appearance were considered to be plasma cells.

Invertebrate blood was obtained by puncturing a blood vessel and allowing blood to drip directly onto a siliconed glass slide. The smear was fixed immediately in osmic acid or formalin vapor. Blood of vertebrates was smeared on glass slides and dried. Spleens and bone marrows were sectioned and exposed surfaces lightly touched to glass slides which were dried. All preparations were stained with Wright-Giemsa and examined using oil immersion lens. A few wet preparations were examined with the phase contrast microscope. One to five specimens of each form were studied.

The following invertebrates were examined: *Arenicola cristata, Loligo pealii, Cambarus limosus, Homarus americanus, Cancer borealis, Libinia emarginata, Limulus polyphemus,* and *Hadrurus arizonensis.* No cells recognizable as plasma cells were found in the blood of any of these.

Among vertebrates, the elasmobranchs and teleosts examined were *Mustelus canis, Squalus acanthias, Carcharhinus obscurus, Raja ocellata, Torpedo nobiliana, Dasyatis centroura, Echeneis naucrates, Lophius piscatorius, Tautoga onitis,* and *Pseudopleuronectes americanus.* Plasma cells were detected in spleens but not in blood of all species.

Two amphibia were studied. No plasma cells were found in blood or spleens of *Rana catesbiana* or *Rana pipiens.* However, bone marrow of *Rana catesbiana* did contain plasma cells. Satisfactory marrow was not obtained from *Rana pipiens.*

These observations demonstrate that cells having morphologic characteristics of human plasma cells exist in lower forms. The phylogenic occurrence of these cells is being correlated with serum electrophoretic studies in an attempt to determine relationships between plasma cells and gamma-globulin.

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CHEMICAL ANALYSES OF ANTERIOR AND POSTERIOR BLASTOMERES OF CIONA INTESTINALIS

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Segregations of specific cytoplasms occur during early cleavage of the ascidian egg (Conklin, 1905) of which the most obvious is the localization of the myoplasm, presumptive for larval musculature, in the posterior cells at the four-cell stage. Cytological (Meves, 1913; Duesberg, 1915; Conklin, 1931) and cytochemical (Ries, 1937) studies indicate the localization of granules, presumably mitochondria, within the myoplasm. Recently Reverberi (1956) followed the distribution of mitochondria during development, using the vital stain, Janus green. The quantitative measurements of cytochrome oxidase in anterior and posterior blastomeres (Berg, 1956) gave a biochemical confirmation of the above studies as regards the localization of mitochondria.

The present study is a continuation of quantitative chemical analyses of anterior and posterior blastomeres from the four-cell stage of *Ciona,* choosing constituents which might be expected to be located on cellular particles. The minute amounts of cytoplasm available require the use of microchemical methods and limit the number of substances which may be studied; however, differences in activities of succinic dehydrogenase, apyrase, acid phosphatase, and ribonucleic acid have been found in homogenates of the two types of cells.

METHODS

Chorions were digested off unfertilized *Ciona* eggs in 3 per cent protease in sea water. The "naked" eggs were washed thoroughly, fertilized, and transferred to agar-coated dishes. At the end of the first cleavage the blastomeres were separated in large numbers by agitation and as each of these cleaved in turn, the anterior and posterior cells were separated with the tip of a fine braking pipette and segregated into different dishes.

The posterior blastomere is recognizable by an elongated shape and a clear cytoplasmic cap (Castle, 1896). These characteristics are transitory and the period for separation is critical; separation before completion of the cleavage furrow results in cytolysis whereas shortly after cleavage identification becomes increasingly difficult due to sphering of the posterior cell. This period was extended by lowering the temperature to 8–10° C. after completion of cleavage, thus prolonging the elongated state of the posterior cell. Furthermore it was discovered that with oblique lighting the posterior cell, even after sphering, exhibits a bright crescentic rim of cytoplasm which is the remnant of the clear cytoplasmic cap. This persists for some minutes after cleavage and greatly extends the length of time during which identification is possible.

Separation of blastomeres was begun as soon as possible after cleavage, using the difference in shapes for identification. As the posterior cells began to round up, an oblique lighting was adopted and separation continued, using the bright rim of the posterior cell as a marker. In this manner several hundred blastomeres could be isolated, a considerable improvement over initial attempts where a maximum of 30–40 were obtained (Berg, 1956).

The desired number of segregated blastomeres was counted and transferred to 0.1-ml. centrifuge tubes by means of a braking pipette. After light centrifugation excess sea water was removed, a few μl. of homogenizing solution were added and the cells homogenized by drawing them in and out of a fine-bore pipette. The homogenization was carried out at 1–2° C., attained by placing the centrifuge tube in a previously chilled copper block. All micromethods were spectrophotometric, using the Beckman spectrophotometer adapted for the use of microcuvettes (Lowry and Bessey, 1946).

Succinic dehydrogenase activity in homogenates was measured by the method of Cooperstein *et al.* (1950). The blastomeres were homogenized in 1 μl. of 0.19 M sodium succinate; 5 μl. of 0.19 M succinate and 2 μl. of 0.03 M sodium cyanide were added and the mixture transferred to 45 μl. of 2×10^{-5} cytochrome *c* contained in a microcuvette. All solutions were buffered with 0.04 M phosphate buffer at pH 7.4. The reduction of cytochrome *c* was followed at 550 mμ for three minutes at the end of which time a few grains of sodium hydrosulfite were added to completely reduce the cytochrome *c*. A semi-logarithmic plot of the readings, after substraction of the optical density of reduced cytochrome *c*, gave a straight line from which a velocity constant, (Δlog ferricytochrome c/ΔT), could be calculated. Succinic dehydrogenase activities were expressed as velocity constants for rates of reduction of cytochrome *c*.

Acid and alkaline phosphatases and apyrase were measured by micromethods described by Lowry *et al.* (1954). For acid phosphatase 10 μl. of substrate (8 mM disodium p-nitrophenyl phosphate in 0.05 M succinate buffer, pH 5) were mixed with the homogenate. After thirty minutes of incubation at 25° C., 45 μl. of 0.1 N NaOH were added, with immediate mixing, and read at 410 mμ within thirty minutes.

Alkaline phosphatase was measured in a similar manner except that a buffer (2 amino-2 methyl-1-propanol) at pH 10 was used with an incubation period of one hour. It was not necessary to carry out protein precipitations in determinations of either acid or alkaline phosphatase. Blanks were prepared by separate incubation of substrate and homogenate with mixing just before addition of NaOH.

Apyrase measurements were made by homogenizing the cells in 0.75 per cent sodium desoxycholate, a procedure which considerably increases the enzyme activity presumably due to particle breakdown. Ten μl. of substrate (2.5 mM adenosinetriphosphate in 0.05 M tris hydroxy-amino methane at pH 8.0) were added to the homogenate and the mixture incubated for one hour at 25° C. Protein was precipitated by adding 2 μl. of 30 per cent trichloracetic acid and, after centrifugation, the supernatant was transferred to another tube with 100 μl. of molybdate-ascorbic acid reagent and read at 870 mμ. Blanks were prepared by separate incubation of substrate and homogenate with mixing at the time of addition of trichloracetic acid. Both apyrase and acid phosphatase activities were expressed as optical densities after subtraction of blank values.

Ribonucleic acid was determined by a micromethod based on the procedure of Ogur and Rosen (1950). Cells were extracted with 60 μl. of cold 70 per cent alcohol for 5–10 minutes followed by extraction with 60 μl. of warm alcohol-ether (3:1). After a few minutes extraction with cold 0.1 *M* perchloric acid, a final extraction with 45 μl. of 1.0 *M* perchloric acid was carried out for 48 hours.

A typical absorption curve for ribonucleic acid was obtained with the latter extract and the optical density at 260 mμ was used as a measure of the amount. Repeated test extractions with 70 per cent alcohol, alcohol-ether, and 0.1 *M* perchloric acid demonstrated that these solutions removed, within a few minutes, all amino acids, polypeptides and acid-soluble substances which absorb at 260 mμ. Although most extractions were carried out for a longer time, nearly all the ribonucleic acid was removed within 24 hours.

Protein was measured by the method of Lowry *et al.* (1951). The cells were placed in a 0.5-ml. test tube and 100 μl. of alkaline copper solution added and mixed. After 10 minutes, 10 μl. of diluted Folin reagent were added with immediate mixing and the sample read at 750 mμ. Addition of the Folin reagent is critical and the reliability of color development depends upon the rapidity and effectiveness of the mixing. On a micro scale this is difficult to control and the resulting variability seriously limits this method.

Calibration curves (Fig. 1) were made for each of the micromethods. Fertilized eggs, with the chorions removed, were used for these tests and as shown in Figure 1 the optical density measurements, or velocity constants for succinic dehydrogenase, are proportional to enzyme activities as represented by the number of eggs. The optical densities at 260 mμ of perchloric acid extracts, representing amounts of ribonucleic acid, are proportional to the number of eggs extracted.

Results

Anterior and posterior blastomeres of *Ciona* were separated and kept at 1° C. in agar-coated dishes until ready for counting and transference to the reaction tubes. From the calibration curves (Fig. 1) an estimation was made of the number of blastomeres necessary in each test for reliable measurements of the constituent. Thirty to forty cells in each tube were sufficient for enzyme and protein measurements, whereas nearly one hundred were required for a reliable measure of ribonucleic acid.

All determinations of enzymes, ribonucleic acid, and proteins in anterior and posterior blastomeres were paired; *i.e.,* two reaction tubes were used, one containing anterior cells, the other containing an equal number of posterior cells. The analyses were then carried out simultaneously. Due to the variability of results obtained with microchemical methods it was necessary to repeat the analyses a number of times and, as each experiment was on eggs from different animals and carried out under slightly different conditions, the data are expressed as ratios of activities or concentrations for each paired experiment. The average of these ratios is used to summarize the results although statistical significance was calculated directly from the paired series.

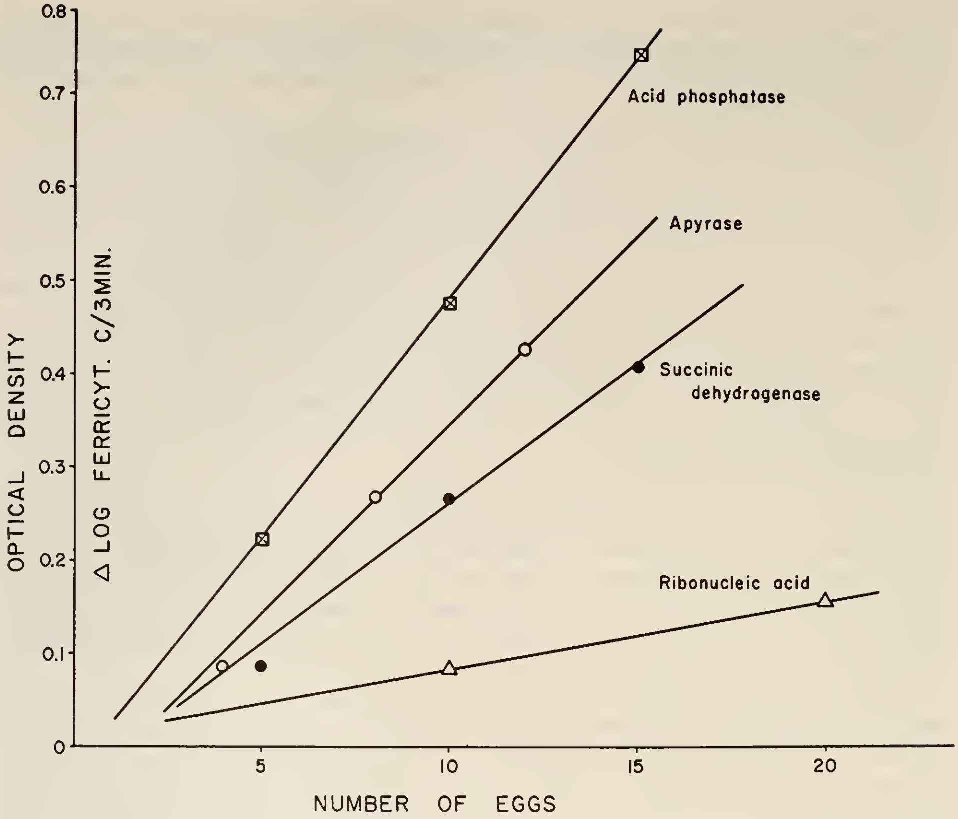

FIGURE 1. Acid phosphatase (activity expressed as optical density), apyrase (optical density), succinic dehydrogenase (Δ log ferricytochrome *c*/3 minutes) and ribonucleic acid (optical density) in homogenates of *Ciona* eggs.

The data for eighteen paired measurements of acid phosphatase in anterior and posterior cells are summarized in Table I. The anterior cells contain 12 per cent more of this enzyme than posterior cells, a difference which is significant at the one per cent level. A number of these experiments were carried out using sodium desoxycholate in the homogenization medium, with no detectable increase in enzyme activity.

TABLE I

Acid phosphatase, expressed as optical densities, in homogenates of anterior and posterior cells of Ciona

Number of tests	Acid phosphatase in anterior cells	Acid phosphatase in posterior cells	Average ratio of paired determinations (ant./post.)
18 (25–40 cells for each test)	0.318	0.287	1.12 ± 0.03

A homogenate of thirty or more *Ciona* eggs was necessary to obtain even a detectable activity of alkaline phosphatase and accordingly it was not feasible to measure the activity of this enzyme in isolated blastomeres. The very low alkaline phosphatase activity in early cleavage stages is also characteristic of the mollusk *Mytilus edulis* and the sea urchin *Strongylocentrotus purpuratus.*

TABLE II

Succinic dehydrogenase, expressed as velocity constants for reduction of cytochrome c, in homogenates of anterior and posterior blastomeres

Number of cells	Succinic dehydrogenase in anterior cells	Succinic dehydrogenase in posterior cells	Ratio (ant./post.)
45	0.048	0.071	0.68
40	.033	.062	.53
45	.025	.070	.36
40	.043	.071	.61
45	.042	.089	.47
		Average	0.53 ± 0.05

As would be expected on the basis of earlier measurements of cytochrome oxidase (Berg, 1956), succinic dehydrogenase activity is greater in posterior cells. The average of five experiments shows that homogenates of posterior cells contain twice as much of this enzyme as anterior cells (Table II).

TABLE III

Apyrase, expressed as optical densities, in homogenates of anterior and posterior blastomeres

Number of blastomeres	Apyrase in anterior cells	Apyrase in posterior cells	Ratio (ant./post.)
44	0.286	0.342	0.84
66	.334	.451	.74
50	.164	.260	.63
60	.230	.352	.65
30	.229	.342	.67
30	.269	.328	.82
30	.269	.412	.65
30	.169	.240	.70
40	.282	.471	.60
		Average	0.70 ± 0.03

Apyrase measurements, summarized in Table III, show that the activity of this enzyme in homogenates of anterior cells is 70 per cent that in homogenates of posterior cells. In adult tissues (Frank *et al.,* 1950) and in amphibian embryos (Barth and Jaeger, 1947) apyrases with different pH activity characteristics have been extracted and thus for quantitative measurements of apyrases from different sources it is necessary to determine pH-activity curves. Accordingly an attempt was made to determine the effect of pH on apyrases from anterior and posterior cells.

Succinate, tris-maleate, and ammediol buffers were used to cover the pH range from 5 to 9.3; pH values were checked with a glass electrode on mixtures prepared on a macroscale exactly as used for microanalyses. Apyrase activities were determined simultaneously at four different pH's in a paired series with thirty anterior or posterior blastomeres in each reaction tube. It was not feasible to cover the entire pH range in any one test; accordingly it was necessary to overlap pH values in different experiments. The considerable variability of determinations was probably in part due to the necessity of constructing the curves from data obtained on different batches of eggs.

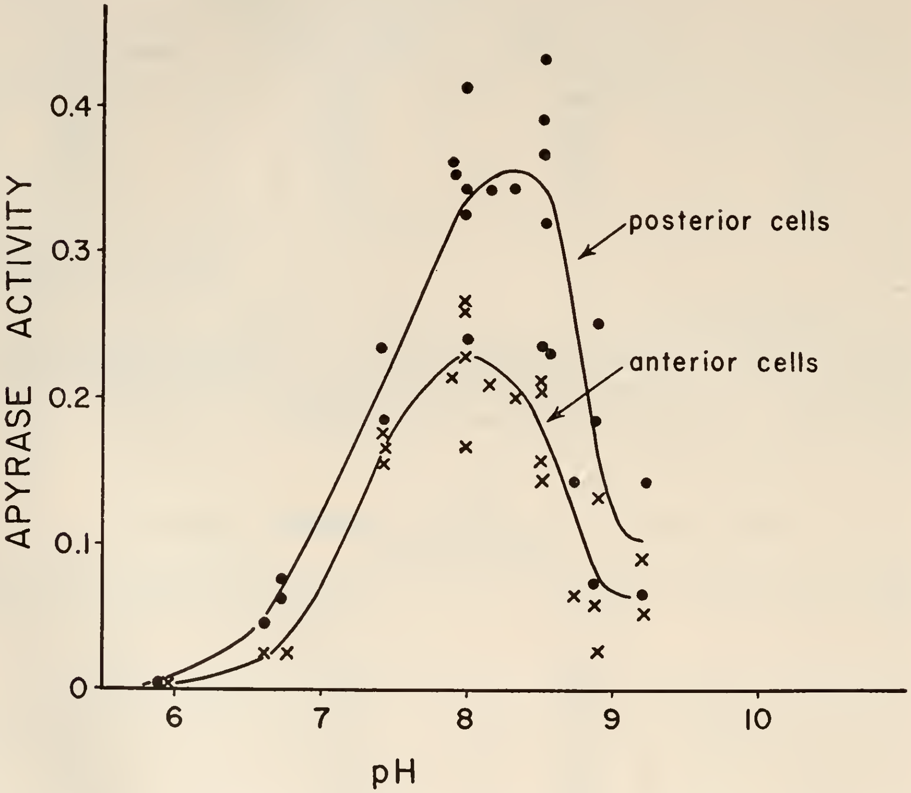

FIGURE 2. Apyrases in homogenates of anterior and posterior blastomeres; pH activity curves.

The pH-activity curves of apyrases from anterior and posterior cells (Fig. 2) are very similar although the optimum for posterior cell apyrase seems more alkaline. The difficulties of obtaining a pH-activity curve on such minute amounts of enzyme are so considerable that this slight difference cannot be considered significant.

The data for paired ribonucleic acid extractions, listed in Table IV, indicate that anterior cells contain 9 per cent less ribonucleic acid than posterior cells, a difference which is significant. This was at first thought to be due to a slight volume difference between anterior and posterior cells. Visual observation of the four-cell stage often gives the impression that the posterior cells are larger. However, careful measurements of volumes by different methods failed to show any significant difference in size.

Diameters of blastomeres, with subsequent cleavages prevented by KCN or fixation in trichloracetic acid, were measured with an ocular micrometer. The average volume of 85 anterior blastomeres, as calculated from diameter measurements with cleavage inhibited by 3×10^{-4} M KCN, was 97 per cent that of 75 posterior cells. The average volume of 134 anterior cells, after fixation in 2 per cent trichloracetic acid, was 98 per cent that of 178 posterior cells. Although with both types of measurements the average volume of the anterior cells was slightly less, statistical analyses of the data failed to show significant differences of these values.

TABLE IV

Ribonucleic acid, expressed as optical densities at 260 mμ, extracted from anterior and posterior blastomeres

Number of blastomeres	RNA in anterior cells	RNA in posterior cells	Ratio (ant./post.)
130	0.285	0.308	0.93
140	.230	.272	.85
114	.216	.240	.90
94	.202	.234	.86
112	.302	.301	1.00
64	.160	.170	.94
70	.154	.200	.77
130	.268	.295	.91
75	.221	.215	1.03
100	.140	.146	.96
120	.271	.320	.85
100	.237	.252	.94
		Average	0.91 ± 0.02

An additional determination of volumes was made indirectly by measuring relative amounts of protein in the two types of cells using a micromethod (Lowry *et al.*, 1951). Twenty-four paired measurements of protein, using 15–40 cells in each test, gave an average protein ratio (ant./post.) of 0.99 ± 0.046. The difference in ribonucleic acid content thus cannot be due to a volume difference and must represent a slight localization of this constituent.

DISCUSSION

A localization of indophenol blue oxidase, presumably cytochrome oxidase, and succinic dehydrogenase in the ascidian embryo has been demonstrated by cytochemical methods (Ries, 1937; Reverberi and Pitotti, 1939; Mancuso, 1952). The microchemical measurements of cytochrome oxidase (Berg, 1956) and those of succinic dehydrogenase in the present study confirm the above cytochemical observations as to the localization of these enzymes in an early cleavage stage.

Information on the intracellular localization of enzymes in the ascidian egg is lacking; however a possible interpretation of the distribution of cytochrome oxidase, succinic dehydrogenase, and apyrase is that they are localized in mitochondria which are unequally distributed in the early cleavage blastomeres. There is considerable evidence that these enzymes are intracellularly located in mitochondria of a variety of cells (reviewed by Schneider and Hogeboom, 1951, 1956). A localization of mitochondria in the myoplasm of the ascidian egg has been demonstrated by cytological methods (Meves, 1913; Duesberg, 1915) and more recently by vital staining with Janus green (Reverberi, 1956). Reverberi (1957a) also presents evidence that cytochrome oxidase is intracellularly localized in the Janus green-staining mitochondria.

It is doubtful that the granules described by Duesberg (1915) and Conklin (1931), classified by them as mitochondria according to morphological and staining criteria, are responsible for the unequal distribution of enzymes. The granules in *Ciona* and "yellow mitochondria" in *Styela* are displaced by centrifugation to the centripetal pole (Conklin, 1931) whereas in centrifuged eggs the oxidase reactions are lacking in this region (Ries, 1939). The yellow granules in *Styela* may be displaced by light centrifugation without altering the localization of the oxidase reactions (Ries, 1942). In centrifuged homogenates of *Ciona* eggs cytochrome oxidase is found in the heavier fraction (Berg, 1956). Furthermore Reverberi (1957a) describes rod-like mitochondria, stainable with Janus green, which, in the centrifuged egg, collect at a different location than the granules described by Duesberg and Conklin. It appears probable, as Reverberi (1957a) also suggests, that several types of mitochondria are localized in the myoplasm of the ascidian embryo.

The intracellular localization of apyrase is probably to some extent within mitochondria, as has been shown for other types of cells (Schneider and Hogeboom, 1951, 1956), and the higher apyrase content of posterior cells most likely results from the segregation of mitochondria into these cells. The average ratio of activities in the two types of cells differs significantly from those for cytochrome oxidase or succinic dehydrogenase, which is interpreted as due to a more heterogeneous intracellular localization of this enzyme as compared to the oxidative enzymes.

Barth and Jaeger (1947) demonstrated that apyrases with different pH activity curves are present in several protein fractions of the amphibian embryo. A similar fractionation of proteins and measurements of the associated apyrases were not possible on *Ciona* blastomeres, due to the minute amounts of material available for analyses. The measurements made therefore represent total apyrase activities of the homogenates and, although there may be qualitatively different apyrases, the similarity of the pH-activity curves for anterior and posterior blastomeres indicates that segregation of this enzyme at the second cleavage is mainly quantitative.

Although acid phosphatase has been found to be intracellularly localized in small mitochondria of adult cells (Appelmans *et al.*, 1955), in the present experiments the opposite distribution of oxidative enzymes and acid phosphatase suggests a non-mitochondrial localization of the latter. It is possible that the localization of acid phosphatase may be a consequence of an unequal distribution of mitochondria. Thus a 2:1 distribution of mitochondria, as indicated by enzyme analyses, might cause displacement of a non-mitochondrial constituent into anterior cells.

Without information on the intracellular localization of ribonucleic acid in the ascidian egg, little can be said regarding the higher ribonucleic acid content of the posterior cells. A factor other than mitochondrial segregation may be involved since mitochondria have a low content of ribonucleic acid (Schneider and Hogeboom, 1951, 1956).

The results are not due to volume differences since extensive measurements, discussed previously, of diameters and total protein in the two types of cells failed to demonstrate any significant differences in volumes. A differential solubility of ribonucleic acid in anterior and posterior blastomeres might lead to erroneous results; however, there is no indication of this, in that continuous extraction for four days with perchloric acid did not change the ratio of amounts extracted.

These quantitative analyses do not, of course, give any information as to the significance of the chemical differences in subsequent differentiation. A few preliminary experiments of rearing embryos in graded concentrations of KCN failed to show, by visual observation of whole embryos, any obvious differential effects on differentiation of anterior and posterior cells. Recently, however, Reverberi (1957b) has shown specific effects of sodium azide, malonate, and selenite on differentiation of the musculature of ascidian larvae, an effect presumably due to blocking activities of mitochondrial enzymes. Previously Ries (1939), by displacement of cytoplasmic areas with centrifugation, had concluded that the presence of the oxidative enzymes was essential for muscle differentiation.

Although localization of oxidases may be of significance in subsequent differentiation of ascidian embryos and several other mosaic forms (*Tubifex,* Lehmann and Wahli, 1954; *Nereis*, Reverberi and Pitotti, 1940; *Myzostoma,* Pitotti, 1947), this is not a common process during cleavage of all mosaic eggs.

Quantitative measurements of cytochrome oxidase in AB and CD blastomeres of *Mytilus edulis* indicated no unequal distribution of the enzyme in this mosaic egg (Berg, unpublished). First-cleavage blastomeres were isolated by previously described methods (Berg, 1950) and cytochrome oxidase measured by a microspectrophotometric method (Berg, 1956). Twelve paired measurements of the enzyme in homogenates of isolated blastomeres gave an average ratio of enzyme activity for the two types of cells (AB/CD) of 0.98 ± 0.03 after correction for volume differences.

Furthermore cytochemical tests for oxidative enzymes failed to reveal segregation of these enzymes during early cleavage of *Sabellaria* (Raven *et al.,* 1950), *Chaetopterus* and *Pomatocerus* (Ries, 1937), *Hydroides* (Reverberi and Pitotti, 1940) and *Limnaea* (Raven, 1946).

I am indebted to Professor Martin W. Johnson of the Scripps Institution of Oceanography, University of California, La Jolla for generously providing space and facilities in his laboratory where a portion of this work was carried out.

SUMMARY

Anterior and posterior blastomeres of the four-cell stage of *Ciona* were separated for quantitative microchemical analyses of succinic dehydrogenase, apyrase, acid and alkaline phosphatases, and ribonucleic acid. Larger amounts of succinic dehydrogenase, apyrase and ribonucleic acid were found in homogenates of posterior cells whereas acid phosphatase activity was higher in anterior cells. The pH-activity curves of apyrases from anterior and posterior cells are similar, indicating a quantitative segregation of this enzyme. The unequal distribution of succinic dehydrogenase, apyrase and, possibly indirectly, acid phosphatase, is probably the result of a segregation of mitochondria.

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THE EFFECTS OF SOME DEVELOPMENTAL INHIBITORS ON THE PHOSPHORUS BALANCE OF AMPHIBIAN GASTRULAE

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Considering the available evidence, it is possible that the morphogenetic movements of gastrulating amphibian embryos are energetically coupled to exergonic metabolic processes, and it is reasonable to assume that the coupling is mediated by energy-rich phosphate-bonds. On this basis, it is possible to propose explanations for the well-known inhibitory effects upon gastrular development of such agents as anaerobiosis, azide and dinitrophenol (Ornstein and Gregg, 1952; Gregg and Ornstein, 1953), for all of these are believed to dissever or restrict energetic couplings: anaerobiosis by switching out the aerobic exergonic processes of the Krebs cycle, azide perhaps by promoting the immediate remineralization of newly esterified phosphorus in the Embden-Meyerhof system (Spiegelman, Kamen and Sussman, 1948), and dinitrophenol perhaps by promoting the catalytic remineralization of esterified phosphorus by mitochondrial dephosphorylases (Hunter, 1951) or by direct "quenching" of energy-rich phosphate bonds (Middlebrook and Szent-Györgyi, 1955). If the energetic demands of gastrular movements are at all appreciable, then embryos treated with such agents might be expected to exhibit decreases in their stores of esterified phosphorus, accompanied by corresponding increases of their inorganic phosphorus contents. The experiments reported in the sequel are intended to test this proposal.

METHODS

Obtaining and rearing embryos. Fertilized eggs were obtained by stripping eggs from gravid *Rana pipiens* females into suspensions of active sperm (*R. pipiens* or *R. sylvatica*). After about two hours, the clutches of embryos were cut with scissors into small groups, dispersed thinly among several finger bowls, and reared at a temperature of 14–15° C. until required for use. The medium in the bowls, changed daily, was 10% amphibian Ringer's solution without phosphate or bicarbonate. Just before use, the embryos were freed of their jelly-coats with forceps. Their developmental stages were determined by reference to the tables of Shumway (1940).

Treatment with inhibitors. Solutions of sodium azide and 2, 4 dinitrophenol were prepared by dissolving weighed samples in aliquots of the same medium in which embryos were reared. The treatment consisted in placing 25 or 30 Stage 10 embryos in a covered stender dish containing 5 or 10 ml. of inhibitor solution for 24 hours at 14–15° C. At the end of this period their developmental stages were noted, then they were washed rapidly with distilled water and lyophilized (see below). Afterward, they were dry-stored in the freezing compartment of a refrigerator until phosphorus analyses could be made, usually within two or three days.

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Anaerobiosis. For each experiment, 25 or 30 Stage 10 embryos were put into an Erlenmeyer flask fitted with a two-hole rubber stopper carrying a gas-inlet tube dipping into the medium in the flask (50 ml. of 10% amphibian Ringer's solution, without phosphate or bicarbonate) and a gas-outlet tube. Nitrogen alone, or 95% N_2 : 5% CO_2 (both previously deoxygenated over hot copper), or hydrogen, was then bubbled through the flasks for one hour, after which the inlet and outlet tubes were closed off with pinch clamps. Controls were prepared similarly, except that air, instead of nitrogen or hydrogen, was bubbled through the medium. After 24 hours (at 14–15° C.), the embryos were removed from the flasks, quickly staged, washed in distilled water and lyophilized. These steps preliminary to freeze-drying were carried out as rapidly as possible to prevent the occurrence of aerobic recovery processes. As before, frozen-dried embryos were stored for a short time, if necessary, in the freezing compartment of a refrigerator.

(The authors are grateful for extensive assistance from Dr. Sasha Malamed in this part of the work.)

Lyophilizing. Embryos were dried *in vacuo* in the frozen state with the help of an all-metal apparatus of conventional design built by Mr. Andrew Pfeiffer, Old Lyme, Connecticut. Washed embryos were placed with a minimum amount of distilled water in 10 × 75 mm. Pyrex test tubes which were then partly immersed in a Cellosolve-dry ice slush. After one or two minutes, the tubes were transferred to the drying apparatus. Drying was usually complete in three hours. Samples for chemical analysis were weighed out as rapidly as possible in a closed balance containing a silica-gel desiccant wafer, since lyophilized amphibian embryos are quite hygroscopic.

Phosphorus analyses. Total phosphorus (P_T), total acid-soluble organic phosphorus (P_{AO}) and inorganic phosphorus (P_I) were estimated, in micro- and semimicro amounts, using the methods of Lowry *et al.* (1954). Since we made no essential departures from their recommendations, the reader is referred to their paper for procedural details. The results of analyses are expressed as micrograms P per milligram dry weight of embryo.

Discussion of Results

Azide and dinitrophenol. At a temperature of 14–15° C., normal *R. pipiens* embryos will develop from Stage 10 (early gastrula) to Stage 12 (late gastrula) in about 24 hours. In the presence of inhibitors, morphogenesis may be reduced in amount or abolished altogether, with varying degrees of recovery following the cessation of inhibitory treatment (Table 1).

After 24 hours in 10^{-5} M azide, *R. pipiens* embryos immersed at Stage 10 will have reached early Stage 12, with no observable after-effects. Embryos similarly treated with 10^{-4} to 10^{-3} M azide will have gastrulated only partially, with after-effects ranging from slight to severe. In 10^{-2} M azide, no gastrulation occurs, and the after-effects are very severe.

After 24 hours in 10^{-7} M 2, 4 dinitrophenol, *R. pipiens* embryos immersed at Stage 10 exhibit no developmental peculiarities, and there are no detectable after-effects of this treatment. In 10^{-6} to 10^{-5} M dinitrophenol development is usually retarded in various degrees and the after-effects range from moderately severe to complete failure of recovery. Dinitrophenol in concentrations of 10^{-4} M, or greater, inhibits gastrulation altogether, and no recovery has yet been observed.

TABLE I

Development of R. pipiens embryos after treatment with azide or dinitrophenol, 14°–15° C. Stage 10 jelly-free embryos were immersed in inhibitors for 24 hours, then washed daily in 10% amphibian Ringer's solution until controls reached Stage 21. Sc_{12} *= stage of treated embryos after 24 hours when controls are in Stage 12.* Sc_{21} *= stage of treated embryos when controls are in Stage 21*

Inhibitor	Molar concentration	Sc_{12}	Sc_{21}	Morphological condition
Azide	10^{-5}	12^-	21	Normal
	10^{-4}	$10\frac{1}{2}$–12	20	Mostly normal. Gills underdeveloped but with circulation
	10^{-3}	10–11	18–20	Thickened tail buds, swellings on flanks, underdeveloped gills with circulation
	10^{-2}	10	18–19	Large yolk plugs, spina bifida, swellings on flanks, muscular response
2,4 Dinitrophenol	10^{-7}	12	21	Normal
	10^{-6}	11–12	11–18	Most in Stage 17–18, fairly normal, some with spina bifida
	10^{-5}	10^+–11^-	10^+–16	Most in Stage 10^+–11^-. Others misshapen, with large yolk plugs, whitish "bloom" on surface coat
	10^{-4}	10	10	"Bloom"
	10^{-3}	10	10	"Bloom"

Thus, it would seem that embryos which have been *totally* blocked with azide can afterwards attain a considerable degree of morphological maturity, something which is apparently denied embryos similarly blocked with dinitrophenol. Just possibly, however, this difference between azide and dinitrophenol may be less related to specific differences in their chemical activities than to differences in their separate abilities to pass outwards through the vitelline membrane. For, we found that even repeated washing fails to remove all of the dinitrophenol from treated embryos; part of it, at least, remains visibly concentrated in the perivitelline fluid. Whether there is a similar retention of azide we do not know, since solutions of this inhibitor are colorless.

We turn now to discuss the effects of such treatments upon the phosphorus balance of amphibian gastrulae.

TABLE II

Developmental stage and phosphorus balance of R. pipiens gastrulae after 24 hours exposure to sodium azide, 14–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations. Parenthesized numerals designate numbers of experiments upon which values are based

Molar conc. azide	Shumway stage	P_T	P_{AO}	P_I
0	10	13.0 ± 0.4 (3)	0.59 ± 0.09 (3)	0.12 ± 0.02 (3)
0	12–12^+	12.9 ± 0.7 (3)	0.59 ± 0.11 (3)	0.13 ± 0.02 (3)
10^{-5}	12^-	13.0 ± 0.3 (2)	0.65 (1)	0.16 ± 0.03 (2)
10^{-4}	$10\frac{1}{2}$	12.8 ± 0.5 (3)	0.43 ± 0.02 (2)	0.23 ± 0.02 (3)
10^{-3}	10^+	13.1 ± 0.4 (2)	0.44 ± 0.04 (2)	0.29 ± 0.05 (2)
10^{-2}	10	13.2 ± 0.5 (2)	0.44 ± 0.01 (2)	0.30 ± 0.01 (2)

Table II shows that the total phosphorus content of *R. pipiens* gastrulae, exposed for 24 hours to 10^{-5} to 10^{-2} *M* azide, is the same as that of untreated control gastrulae. This is an important point because (together with the data in Table I) it suggests that such embryos are practically undamaged, otherwise a certain amount of leakage of phosphorus might be expected. The other two phosphorus fractions, however, exhibit changes, for as the concentration of environmental azide is increased, there is an increase of the inorganic phosphorus content of treated embryos, accompanied by a quantitatively similar decrease in the level of acid-soluble organic phosphorus. Furthermore, as the concentration of environmental azide is progressively elevated, there is a parallel increase in the severity of gastrular retardation.

The data summarized in Table III show that a similar set of results may be obtained with 2, 4 dinitrophenol at concentrations of 10^{-5} *M*, or lower. At concentrations greater than 10^{-5} *M*, however, gastrulae are damaged to such an extent that they begin to leak phosphorus; this is clearly shown by a correlated decline in the levels of all three phosphorus fractions.

Finally, it should be noted that the gastrulation of untreated control embryos is unaccompanied by any significant alterations of phosphorus balance.

TABLE III

Developmental stage and phosphorus balance of R. pipiens gastrulae after 24 hours exposure to 2,4 dinitrophenol (DNP), 14–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations

Molar conc. DNP	Shumway stage	P_T	P_{AO}	P_I	No. expts.
0	10^{-}–10	13.1 ± 0.7	0.59 ± 0.07	0.13 ± 0.01	2
0	12	13.1 ± 0.1	0.58 ± 0.04	0.13 ± 0.01	2
10^{-6}	12	12.9 ± 0.1	0.60 ± 0.09	0.17 ± 0.05	2
10^{-5}	10^{+}–11^{-}	12.6 ± 0.2	0.45 ± 0.01	0.31 ± 0.02	2
5×10^{-5}	10^{-}	11.9	0.34	0.22	1
10^{-4}	10^{-}–10	12.4 ± 0.3	0.19 ± 0.09	0.08 ± 0.08	2

Those results suggest the following interpretation. If the movements of gastrulation of normal untreated embryos demand an available supply of phosphate-bond energy, the resulting draughts upon esterified phosphorus are immediately reimbursed, and the phosphorus balance is steadily maintained. This result is in full agreement with the data of Barth and Jaeger (1947), summarized in their Table 1. It is also consistent with the view that gastrulation is a complex of morphogenetic movements whose execution requires no expenditure of energy. The results with azide and dinitrophenol suggest the contrary, however, because of the correlation between the presence of these inhibitors, the reduction of esterified phosphorus, the elevation of inorganic phosphorus, and the retardation of gastrular movements. For it is difficult to explain this correlation except by assuming that in the presence of inhibitors the production of esterified phosphorus is uncoupled from its utilization as a source of morphogenetic energy because it is made available to enzymes catalyzing its remineralization (see the remarks at the beginning of this paper). Of the complex of movements, Gregg and Ornstein (1953) have presented evidence suggesting that epiboly is the most sensitive to treatment with dinitrophenol, while

TABLE IV

Developmental stage and phosphorus balance of R. pipiens gastrulae after 24 hours anaerobiosis, 14°–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations. P values in A and B are listed separately in order that the former may be compared with those obtained from hybrid embryos prepared from the same five clutches of eggs (Table V)

Treatment		Shumway stage	P_T	P_{AO}	P_I	No. expts.
A	Control	10	13.5 ± 0.4	0.58 ± 0.04	0.13 ± 0.02	5
	Control	12	13.2 ± 1.0	0.55 ± 0.03	0.13 ± 0.01	5
	Nitrogen	11	13.5 ± 1.1	0.56 ± 0.05	0.15 ± 0.01	5
B	Control	10^+	12.4	0.58	0.12	1
	Control	12	13.00	0.52	0.13	1
	Nitrogen	11	12.9	0.52	0.18	1
C	Control	10	12.4	0.58	0.13	1
	Control	12^-	12.5	0.60	0.13	1
	Hydrogen	11^-	12.5	0.64	0.18	1

epiboly and notochordal elongation are most affected by the presence of azide; but the exact relations of their results to the present ones are yet to be worked out.

Anaerobiosis. Table IV summarizes the results of experiments designed to show the effects of 24 hours anaerobiosis on the phosphorus balance of *R. pipiens* gastrulae. Embryos are morphologically retarded under these conditions, but, in general, there is no alteration of the phosphorus balance other than a slight elevation of the inorganic phosphorus level. The total phosphorus and acid-soluble organic phosphorus levels are unaffected. There is thus a considerable morphogenetic effect of anaerobiosis, apparently unaccompanied by a decrease in the stored phosphate-bond energy potentially available. This conclusion is not in agreement with that of Barth and Jaeger, who found that anaerobioses of 10 to 22 hours duration resulted in a considerable decrease of ADP–ATP phosphorus. Their fractionation procedure is not strictly comparable with ours, however, and the apparent discrepancy cannot be resolved without further investigation.

TABLE V

Developmental stage and phosphorus balance of R. pipiens ♀ × R. sylvatica ♂ gastrulae after 24 hours anaerobiosis, 14–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations. These values should be compared with those in part A of Table IV, obtained from R. pipiens embryos prepared from the same five clutches of eggs

Treatment	Shumway stage*	P_T	P_{AO}	P_I	No. expts.
Control	10	13.2 ± 1.0	0.57 ± 0.04	0.12 ± 0.01	5
Control	12	13.7 ± 0.7	0.55 ± 0.05	0.12 ± 0.01	5
Nitrogen	11	13.6 ± 1.0	0.56 ± 0.05	0.14 ± 0.02	5

* The stages assigned are those of simultaneously developing *R. pipiens* control embryos (Table IV, A).

Embryos in the hybrid *R. pipiens* ♀ × *R. sylvatica* ♂ fail to gastrulate, but remain alive during the whole time required for control *R. pipiens* embryos to reach the hatching stage (Moore, 1946; see review by Gregg, 1957). They are characterized by low respiratory rates and by low rates of aerobic and anaerobic glycolysis. The expectation that they might therefore find it more difficult than normal embryos to maintain esterified phosphorus stores under the stress of anaerobiosis was confirmed by the data of Barth and Jaeger. Our own experiments do not bear out this expectation, for they show (Table V) that hybrid embryos under anaerobiosis do not alter their phosphorus balance to a greater extent than *R. pipiens* controls. But it is not clear that these results are in genuine disagreement with those of Barth and Jaeger, for the reason stated at the end of the preceding paragraph.

SUMMARY

1. *Rana pipiens* gastrulae treated with non-damaging concentrations of sodium azide or 2, 4 dinitrophenol for 24 hours at 14–15° C. exhibit a reversible retardation of morphogenetic movements, a diminished store of acid-soluble organic phosphorus, an elevated content of inorganic phosphorus and an unaltered total phosphorus content.

2. Anaerobiosis for 24 hours at 14–15° C. does not alter the phosphorus balance of *R. pipiens* gastrulae, or of gastrula-arrested hybrids of *R. pipiens* ♀♀ with *R. pipiens* ♂♂, beyond a slight elevation of the inorganic phosphorus level.

3. These results are discussed briefly in respect to the energy-requirements of the morphogenetic movements of gastrulation.

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RESPIRATION OF HOMOGENIZED EMBRYOS: RANA PIPIENS AND RANA PIPIENS ♀ × RANA SYLVATICA ♂[1]

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Embryos belonging to the hybrid *R. pipiens* ♀ × *R. sylvatica* ♂ cleave and blastulate normally, but the normal sequence of gastrulation movements does not occur (Moore, 1946). Such embryos remain alive, but suspended in a morphological state superficially similar to that of a very young gastrula. Before the occurrence of the developmental block, the respiration of hybrid embryos is quantitatively similar to that of normal *R. pipiens* controls, increasing exponentially with age; but for most of the period following its occurrence, their respiration is characterized by a function whose value is a constant (Barth, 1946). For an account of the attempts that have been made to analyze the biochemical and morphogenetic peculiarities of this hybrid, the reader is referred to the review by Gregg (1957).

The problem of explaining the respiratory peculiarities of gastrula-blocked hybrids is of course closely connected with that of constructing a theory to account for the exponential respiratory increase characterizing the pre-hatching development of normal embryos. In connection with this latter problem, suggested explanations have tended to fall into at least two classes: (1) those which postulate an increasingly rapid developmental synthesis of respiratory enzymes or substrates, and (2) those which assume a progressive increase in the structural availability of respiratory enzymes to their substrates. Each of these is supported by at least some of the available evidence. On the assumption that one or both of those types of explanation is well-founded, it is plausible to suggest two corresponding sorts of explanation to account for the post-blastula deficiencies of hybrid respiration: (i) that there is a failure to continue the synthesis of respiratory enzymes or substrates at a sufficient rate, or (ii) there is a failure of some developmental process which normally continues to increase effective contact of respiratory enzymes and their substrates.

In this paper we report the results of some simple homogenization experiments which it is hoped will have some bearing on these various questions.

METHODS

Fertilizing, rearing and staging embryos. For fertilizing and rearing embryos, the following routine was adopted. Two separate sperm suspensions, one of *R. pipiens* sperm and one of *R. sylvatica* sperm, were prepared simultaneously in two fingerbowls, N and H. Half of the ripe eggs from a gravid *R. pipiens* were stripped into N and half into H. After two or three hours, the *R. pipiens* embryos in N were separated with scissors into small groups and distributed among several fingerbowls $N_1, \ldots N_n$; the hybrid embryos in H were similarly dispersed among several bowls $H_1, \ldots H_n$. Embryos in pairs of bowls (N_i, H_i) were reared at

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similar temperatures, ranging from 8° C. to 25° C., as convenient. Thus, the normal embryos in bowl N_i served as controls for the hybrids in the corresponding bowl H_i. The medium in the bowls, 10% amphibian Ringer's solution without phosphate or bicarbonate, was changed daily. At the desired stages, obtained by reference to the tables of Shumway (1940), embryos were freed of their jelly coats with jeweler's forceps, re-staged, and homogenized. Hybrid embryos were assigned the stage-numbers characterizing the developmental stages of normal control embryos.

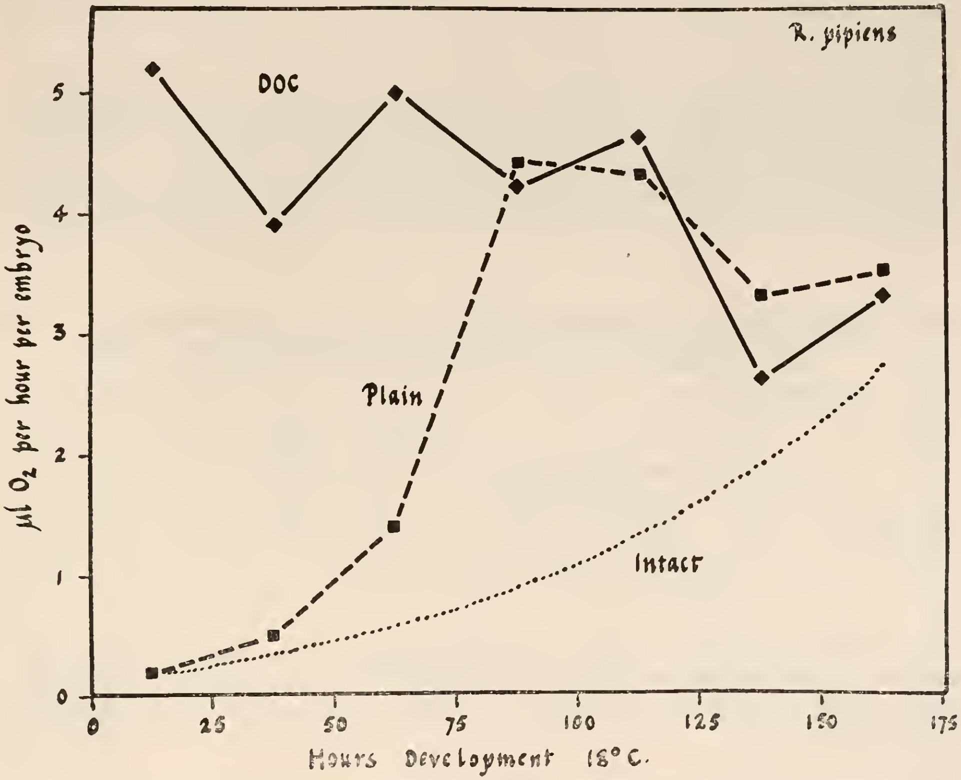

FIGURE 1. Respiration of homogenized *R. pipiens* embryos. Lower curve for intact *R. pipiens* embryos constructed from data of Moog (1944).

Preparation of homogenates. Cell-free breis were prepared by homogenizing batches of *n* jelly-free embryos in 0.05 *n*-ml. aliquots of suitable ice-cold media, using a high-speed homogenizer manufactured by the Lourdes Instrument Corporation. Two sorts of media were used routinely:

(a) 0.01 *M* phosphate buffer made up in 10% amphibian Ringer's solution without phosphate or bicarbonate, pH 6.8–7.0 Breis prepared in this medium will be called *plain-breis.*

(b) Medium (a) with the addition of 0.2% sodium deoxycholate (DOC). Breis prepared in this medium will be called *DOC-breis.* (We are indebted to Dr. W. H. Berg for suggesting the use of deoxycholate.)

Measurement of brei respiration. Within 10 minutes after preparation, 1.0-ml. aliquots of cold homogenate (about 20 embryos) were pipetted into 7-ml. Warburg flasks rigged for the measurement of oxygen uptake. After a period of temperature equilibration in the respirometer bath (about 10 minutes), manometer readings were begun and continued at 6-minute intervals for 45–60 minutes. The temperature of the respirometer bath was controlled at 24° C. The flasks were shaken continuously at a rate of 100–110 complete cycles per minute, at an amplitude of 6–8 centimeters. The rates of oxygen uptake were calculated from the readings taken during the first 30 minutes and are expressed as microliters (μl.) of oxygen per hour per embryo.

Treatment of data. In Shumway's tables, each stage s is correlated with a unique time t(s), namely, the time required for a normal embryo to develop from fertilization to that stage, at 18° C. Furthermore, each t(s) falls in exactly one of the successive 25-hour intervals following the moment of fertilization. We have made use of these correlations in presenting the results of measurements of the respiration of breis made from embryos with different environmental (temperature) histories. Thus, to construct Figure 1 and Figure 2, we have averaged for each 25-hour interval the respiratory rates of breis made from embryos whose stage s has a t(s) in that interval and have plotted the resulting average against the interval's midpoint.

TABLE I

Effect of buffer concentration on respiration of plain- and DOC-breis, R. pipiens, stage 10. After equilibration period in respirometer, deoxycholate in appropriate buffer, pH 6.8–7.0, added from side-arm. Final brei concentration, 20 embryos per ml.

Plain-breis	Buffer concentration, molar	0.025	0.05	0.075	0.1	0.15	0.2
	μl. O_2 per hour per embryo	0.35	0.23	0.43	0.43	0.27	0.19
DOC-breis, 0.2% DOC	Buffer concentration, molar	0	0.01	0.02	0.04	0.07	0.08
	μl. O_2 per hour per embryo	7.8	8.0	7.9	6.4	6.6	6.9

RESULTS

R. pipiens, plain-breis. Reference to Figure 1 will show that the average respiratory rate of plain-breis increases exponentially with the age of the embryos used in their preparation from an initial value of about 0.2 μl. O_2 per hour per embryo to a maximum value in the fourth 25-hour interval of about 4.5 μl. O_2 per hour per embryo, and thereafter tends to decline.

This result is in sharp contrast to that of Spiegelman and Steinbach (1945), who reported that the endogenous respiration of plain-breis prepared from embryos shortly after fertilization is already at a maximum. We are at a loss to explain the discrepancy, for we have been able to elevate the respiration of breis only by treatment with a detergent (see next paragraph), but not by altering the buffer concentration (Table I), nor, in preliminary experiments, by adding glycogen, glucose, hexose diphosphate, adenylic acid, magnesium, or various combinations thereof.

TABLE II

Effect of sodium deoxycholate concentration on respiration of homogenized R. pipiens embryos, stage 10–11. Breis and deoxycholate made up separately in 0.07 M phosphate buffer, pH 6.8–7.0. After equilibration period in respirometer bath, deoxycholate added from side-arm. Final brei concentration, 20 embryos per ml.

Final concentration of sodium deoxycholate, per cent	0	0.1	0.2	0.4	0.8
μl. O_2 per hour per embryo	0.12	2.8	6.6	5.6	4.4

R. pipiens, DOC-breis. The maximum respiratory rate attained by plain-breis, *i.e.*, that exhibited by embryos homogenized during the fourth 25-hour period of development, can be matched by that of deoxycholate-treated breis prepared from embryos of any lesser age (Fig. 1). Plain-breis and DOC-breis prepared from embryos older than this, however, respire at the same rate. The degree of respiratory elevation obtained earlier is a function of the concentration of DOC, 0.2% being the optimal concentration (Table II).

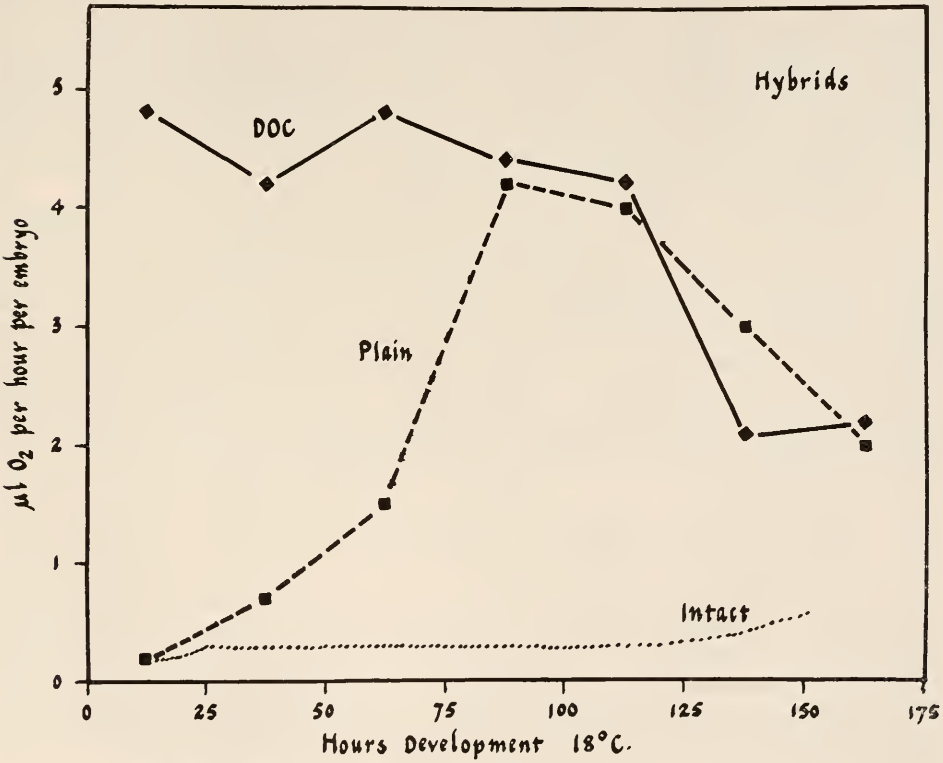

FIGURE 2. Respiration of homogenized gastrula-blocked hybrid embryos (*R. pipiens* ♀ × *R. sylvatica* ♂). Lower curve for intact embryos constructed from data of Barth (1946) and Moog (1944).

The curve for DOC-breis shown in Figure 1 is very similar to that reported for plain-breis by Spiegelman and Steinbach, although the general respiratory level is much higher and begins to decline somewhat later. We do not know if the stimulatory effect of DOC can be obtained with other detergents. However, we have found that plain-breis of early embryos respire at unusually high rates if they are prepared in Waring Blendor vessels freshly washed in Alconox. The effect wears off after several preparations without intervening cleansings with Alconox. This suggests that detergent is trapped in the bearings and leaks out slowly during the preparation of breis, but a systematic study of the question has not been made.

Hybrids, plain-breis and DOC-breis. The results of our measurements of the respiratory rates of plain- and DOC-breis made from hybrid embryos (Fig. 2) can be summarized very briefly: the respiration of such breis is quantitatively similar to that of corresponding breis made from normal control embryos, except perhaps in the seventh 25-hour period when the hybrids are moribund.

Discussion

There is no need to postulate a developmental synthesis of respiratory enzymes or substrates to account for the exponential rise of respiratory rate characterizing the development of normal amphibian embryos; for, as the high respiratory rates of DOC-breis show, there is from the outset of development enough respiratory machinery to support oxidation-rates greater than any exhibited by intact pre-hatching embryos. Similarly, there is no need to assume a synthetic failure to account for the abnormal constancy of post-blastula hybrid respiration, because the experiments with DOC-breis have shown that hybrid embryos at nearly all stages are potentially capable of as much respiration as normal controls.

For normal embryos, we adopt the conclusion of Spiegelman and Steinbach, namely, that the exponential respiratory increase is causally related to morphogenetic changes which progressively facilitate the union of respiratory enzymes and their substrates. Correspondingly, to explain the respiratory constancy of aging post-blastula hybrids, we assume that those changes somehow have been brought to a halt at the commencement of the gastrula stage. What sorts of changes might be involved is at present unknown. The elevation of embryonic respiration obtained by homogenizing in plain phosphate buffer, increasingly extensive as development proceeds, suggests that some cellular structures are becoming increasingly sensitive to mechanical disturbance or to alterations of chemical milieu, and the maximal respiration obtained at all stages by the treatment of breis with deoxycholate suggests a cellular component sensitive to detergent action: in both cases the mitochondria come immediately to mind because of their close association with oxidative enzymes of the Krebs cycle and of the hydrogen transport system, and because they are sensitive to isolative procedures, especially in the presence of deoxycholate (Siekevitz and Watson, 1956). It is reasonable to suppose that the extensive fragmentation of mitochondria by deoxycholate is only a more thoroughgoing version of what happens at any developmental stage to the mitochondria in plain-breis. In any case, it would be very interesting to study the mitochondria of differentiating embryos, and to compare those of hybrids with those of normal embryos.

The decline in the respiration obtainable from breis of late embryos may be explained by assuming, as Spiegelman and Steinbach do, a depletion of endogenous respiratory substrates. In this connection, it is known that only half of the stored carbohydrate available at the time of fertilization remains in normal embryos at the time of hatching, although more than this remains in hybrids of the same age (Gregg, 1948).

SUMMARY

1. The respiration of phosphate-buffered cell-free homogenates made from *R. pipiens* embryos increases exponentially with the age of the embryos up until the time at which they are in the tailbud stage, after which the rate declines.

2. Addition of 0.2% sodium deoxycholate elevates the respiration of homogenized embryos at any pre-tailbud stage to that of tailbud-breis, but has no effect upon that of breis of post-tailbud embryos.

3. The respiration of plain- or deoxycholate-treated breis is at all stages greater than or equal to that of intact embryos.

4. The respiration of breis (plain- and deoxycholate-treated) made from gastrula-arrested *R. pipiens* ♀ × *R. sylvatica* ♂ hybrid embryos is at all non-moribund stages quantitatively the same as that of control breis of normal embryos.

5. The implications of these findings are briefly discussed.

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A RATIONAL APPROACH TO THE PROBLEM OF CANCER CHEMOTHERAPY[1]

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In spite of the fact that over a long period of time, many workers in various parts of the world have been studying the curative effect of various chemical agents on cancer, there has been no very great effort to determine why these agents act as they do, and there is at present but little pertinent information as to the type of action these agents have on the cancer cell.

The problem is complicated by the fact that many of the very agents that have a definite curative effect can also act as carcinogenic agents. This was first noted by Haddow in 1935, and he and his collaborators have written a number of papers on the subject. In his very useful book on the "Biochemistry of Cancer," Greenstein (1954) frequently refers to this phenomenon as the "Haddow paradox." But neither Haddow nor Greenstein has any interpretation of the paradox. In 1951, Haddow in writing about carcinogens and substances which have a therapeutic effect on cancer writes (p. 264): "But in no case—a striking fact—do we know the place in the cell at which they act—whether the cell surface, the cytoplasm, the nuclear membrane, the nucleus itself—or the nature of the receptors with which they combine." And all that Greenstein has to suggest when he considers the problem is that (p. 278) "The capacity for intellectual flexibility combined with scientific care is one of the demands in the field."

In the past, most of the work that has been done on the chemical treatment of cancer and the theory back of such treatment has been done by chemists. And whatever efforts they have made toward interpretation have for the most part been inspired by chemical concepts built around the idea of some disturbance of metabolism. But some of the most powerful chemotherapeutic agents do not affect the growth of the cell. Thus in the presence of nitrogen mustard or its oxygen derivative, Nitromin, cells increase in size but do not divide (Bodenstein, 1947; Friedenwald, Buschke and Scholz, 1948; Sato, Belkin and Essner, 1956).

When a tumor arises in an organ or tissue the appearance of the neoplasm is always accompanied by a great increase in the number of cells that are dividing. Thus for example in the brain, there are normally no mitoses, but in a brain tumor there are great numbers of dividing cells. It is of course possible to believe that some increase in metabolism, or some change in metabolism, is the primary cause that started the tumor to develop. But it is just as possible to assume that the primary factor is an initiation of mitosis and that metabolic changes are a result rather than a cause.

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At the present time, we have a considerable body of information concerning the initiation of cell division, and there is also a satisfactory theory as to why and how cells can be made to divide. Moreover we have information as to the suppression of cell division and the reasons for it. Both the initiation and the suppression of cell division can be understood in terms of the behavior of the protoplasmic colloid, and the changes that occur in it that lead to the formation of the mitotic spindle. For a survey of this knowledge, see Heilbrunn's "Dynamics of Living Protoplasm" (1956). One of the strange facts about protoplasm is that the same agents which suppress its activity can also under certain conditions arouse it to activity. We thus have a parallel to the Haddow paradox, and indeed the initiation of cell division can be regarded as one example of the response to stimulation.

Can we hope therefore to interpret the chemotherapy of cancer and the Haddow paradox on the basis of a colloidal theory? Surely such an approach is worth looking into, especially in view of the fact that other types of interpretation have not as yet been successful.

In a long series of papers, most of them referred to in the book already cited, Heilbrunn and his co-workers have investigated the problem of the initiation and suppression of cell division from the standpoint of the colloidal properties of protoplasm and the changes that the protoplasmic colloid must undergo in order to form a mitotic spindle. The basic colloidal reaction of protoplasm is a clotting reaction similar in many respects to the clotting of blood. The protoplasm is in a state of equilibrium between the various factors which favor or induce clotting and those which prevent it. Before a mitotic spindle can form, a gelation must occur in the protoplasm. This is the mitotic gelation, and it can be induced either by a release of calcium from a bound state in the outer cortex of the cell or by the entrance into the cell of thrombin-like substances. Many agents prevent the mitotic gelation—among them, substances of a heparin-like nature.

Starting out from this point of view, we thought to investigate the action of certain chemotherapeutic agents which have been used in the experimental study of cancer as well as in clinical practice. The first question to be answered is whether or not these agents prevent the mitotic gelation. Then later we will consider the question of the Haddow paradox.

Materials and Methods

The cell we used as a test object was the egg of the worm *Chaetopterus pergamentaceus.* The eggs of this worm can readily be obtained at Woods Hole during the summer months. When the eggs are shed into sea water they are in the germinal vesicle stage. In 7 minutes (at 21° C.) the germinal vesicle breaks down and the first maturation division proceeds as far as the metaphase. Then all mitotic activity ceases until the egg is fertilized. Following fertilization the first maturation division is completed and the second maturation division immediately follows the first. As a result two polar bodies are given off and the egg then prepares for its first cleavage division. The mitotic spindle for this division appears at forty minutes after insemination (at 21° C.) and 50% of the eggs cleave at 56 minutes after insemination. Before the appearance of the mitotic spindle, the viscosity of the protoplasm increases markedly; this constitutes the mitotic gelation. Simultaneously with the appearance of the spindle the protoplasm becomes more fluid again so that at the metaphase the viscosity is again low. Details of these changes are given by Heilbrunn and Wilson (1948). One advantage in using the *Chaetopterus* egg is that in any given lot the course of events in different individual eggs varies but little, and there is almost perfect synchrony.

Viscosity measurements were made with a hand centrifuge. At the present time it is not possible to buy suitable hand centrifuges. The ones we use are made for us by Mr. J. A. Appenzeller, technician of the Zoology Department of the University of Pennsylvania. They are adapted from a hand centrifuge sold by Sears, Roebuck and Co. and intended for the separation of cream from milk. In order to use these cream separators for our purposes it is necessary to fit them with a head which will hold glass tubes. The tubes we use have an outside diameter of 4 or 5 millimeters. When our centrifuges are turned at the rate of one turn of the handle per second, they give a force approximately 9,000 times gravity. We prefer to turn the handle once every two seconds; this gives a force one-fourth as great, that is to say, 2,250 times gravity. With a force of this magnitude, for most of the time between insemination and cleavage, it requires 7 seconds to move the granules in the *Chaetopterus* egg sufficiently so as to give the impression of zones. (The heavier granules move centrifugally and the lighter granules centripetally.) The number of seconds required to produce zoning is taken as an arbitrary viscosity value. At about 27 minutes after insemination (at 21° C.) the viscosity of the protoplasm begins to increase, and by about 30 or 32 minutes the viscosity has increased until it is approximately twice what it was before this mitotic gelation began. The viscosity then stays high until the spindle appears, a matter of about 8 minutes. It is during this time that tests of viscosity must be made if we are to discover if a given substance keeps the protoplasm fluid and prevents the mitotic gelation. These tests must therefore be made rapidly so that it is not possible to obtain definite values. But we can be sure that in the controls mitotic gelation has occurred if the viscosity is high enough so that it requires more than 8 seconds of centrifugal turning to cause an appearance of zones. Actually the viscosity during normal mitotic gelation is 14 in our arbitrary units. In the tables we record the viscosity of the control eggs as more than 8; it almost certainly is 14.

The nitrogen mustard used in our experiments was obtained from Sharp and Dohme in the form of a commercial preparation called Mustargen. This preparation comes in separate sealed vials, each of which contains 10 mg. of nitrogen mustard, that is to say methyl-bis(beta-chloro-ethyl) amine hydrochloride plus 100 mg. of NaCl. The contents of each vial were hastily dissolved in sea water, but because of the presence of the NaCl it was not possible to prepare solutions of high concentration, for such solutions would have been hypertonic and might have masked the effect of the nitrogen mustard.

Through the kindness of Dr. Edward S. Essner we were able to obtain Nitromin, an oxide derivative of nitrogen mustard manufactured by the Yoshitomi Pharmaceutical Industries of Osaka in Japan and distributed by Takeda Pharmaceutical Industries, also of Osaka. Nitromin is methyl-bis(beta-chloroethyl)amine-N-oxide hydrochloride. This compound is less toxic than nitrogen mustard and has been claimed to have better therapeutic value.

In using 6-mercaptopurine, we had difficulty. This substance is scarcely soluble at all in sea water. In order to obtain a solution we dissolved it first in a small amount of normal NaOH. Then strongly acidified sea water was added until a pH

TABLE I

Effect of Mustargen on fertilized Chaetopterus eggs

%	pH	Viscosity at 32–38 min.	% Cleavage
0 (control)		>8	94
0.1	7.5	8 or less	18
0.05		8 or less	20
0.025		8 or less	16
0 (control)		>8	94
0.1	7.5	8 or less	27
0.05		8 or less	32
0.025		8 or less	19

a little higher than that of sea water was reached. At this pH, microscopic observations showed the solution to be full of suspended material, so that we could not be at all certain as to how much of the substance remained in solution. In all of our experiments we were dealing with a saturated solution of unknown concentration, and we have the impression that in sea water 6-mercaptopurine is barely soluble.

RESULTS

When nitrogen mustard is dissolved in sea water the resultant solution has a much lower pH than does sea water. In our first experiments we made no attempt to neutralize the acid in our solutions, and the results of these experiments were therefore discarded. Table I gives the results of two experiments in which the Mustargen solution was made more alkaline by the addition of NaOH solution. In both experiments, the solutions were brought to a pH of 7.5 and were thus still somewhat less alkaline than sea water. However, in preparing dilutions from the 0.1% solution of nitrogen mustard, the dilutions were of course made with sea water so that in the lower concentrations of the drug, the pH was not very different

TABLE II

Effect of Nitromin on fertilized Chaetopterus eggs

%	pH	Viscosity at 31–35 min.	% Cleavage
0 (control)		>8	100
0.4	7.9	8 or less	0.5
0.3		8 or less	4
0.2		8 or less	9
0 (control)		>8	97
0.5	8.0	8 or less	0
0 (control)		>8	97
0.4	7.8	8 or less	0
0.3		8 or less	0
0.2		8 or less	2

from that of sea water. Moreover, a pH of 7.5 has but little effect on the protoplasm of *Chaetopterus* eggs.

The experiments with nitrogen mustard indicate that this substance keeps protoplasm fluid and prevents the mitotic gelation. It thus acts in the same way as do various other antimitotic substances previously studied by us (Heilbrunn and Wilson, 1950a, 1950b, 1956; Heilbrunn, Wilson and Harding, 1951; Heilbrunn, Chaet, Dunn and Wilson, 1954). The mechanism of this action will be discussed later.

Our experiments with Nitromin gave more striking results than those with nitrogen mustard. Like Mustargen, Nitromin when dissolved in sea water causes a substantial reduction in the pH. As before, we added enough NaOH to bring back the solution to a pH like that of sea water. The results obtained with Nitromin are shown in Table II. They show conclusively that this derivative of nitrogen mustard keeps protoplasm fluid and completely prevents the mitotic gelation. Very few of the eggs exposed to rather dilute solutions of Nitromin ever cleave.

As pointed out in the section on Materials and Methods, 6-mercaptopurine is very sparingly soluble in sea water, and sometimes we wondered if any of it went into solution at all. Our results with this substances are not very impressive; they are shown in Table III. The results we did obtain indicate clearly enough that 6-mercaptopurine tends to keep the protoplasm of the *Chaetopterus* egg fluid. This effect is more pronounced when the solution is a little more alkaline, presumably because at the higher alkalinity more of the substance stays in solution. Also at the higher pH the inhibition of cleavage was greater.

TABLE III

Effect of 6-mercaptopurine on Chaetopterus eggs

%	pH	Viscosity at 30–35 min.	% Cleavage
0 (control)		>8	97
0.1	8.25	8 or less	49
0 (control)		>8	98
0.3	8.1	8	99
0.3 (exposure 30 min. before fertilization)	8.1	8	70

Nitrogen mustard, Nitromin and 6-mercaptopurine thus all have the same sort of effect on the protoplasmic colloid. All of them tend to keep the protoplasm fluid and prevent the mitotic gelation. In the past it has been shown many times that the same agents which prevent gelation may, in other concentrations, have quite the opposite effect (for references and discussion, see Heilbrunn, 1956). Fat solvent anesthetics, which keep protoplasm fluid and thus prevent response to stimulation, may in certain concentrations act as stimulating agents and when they do they induce a clotting or gelation of the protoplasm. Now it is proper to consider the prevention of cell division by agents which do not kill the cell as a form of anesthesia or narcosis, and indeed various anesthetic agents do prevent cell division. And the initiation of cell division can be regarded as a response to stimulation. All this being true, might it not be possible to show that with other concentrations of nitrogen

TABLE IV

Effect of ethyl urethane on fertilized Chaetopterus eggs

% Urethane	Viscosity 30–33 min. after fertilization	% Cleavage
0 (control)	>8	100
2	8 or less	0
1.5	8 or less	0
1	8 or less	0

mustard or Nitromin a gelation of the protoplasm could be induced and perhaps also an initiation of cell division? If we could show this, we would have a way of interpreting the Haddow paradox.

We did not attempt to do this experiment with nitrogen mustard, for the preparation of this drug that was available to us—Mustargen—contains ten times as much NaCl as it does nitrogen mustard, and if we made relatively concentrated solutions we would arrive at concentrations of salt which would in themselves cause the initiation of cell division. However, with Nitromin this difficulty does not exist. Accordingly, we tried the effect of a 1% and a 0.5% solution of Nitromin on unfertilized *Chaetopterus* eggs. Both of these solutions caused a marked increase in the viscosity of the protoplasm. In the weaker solution this increase (after 80 minutes) was at least two-fold; in the stronger solution the viscosity increase was even greater and the protoplasm seemed quite solid. In both cases the drug caused a vacuolization of the protoplasm. This is the type of reaction which Loeb (1913) called cytolysis, and it is a reaction commonly produced by agents which initiate division in marine eggs when these agents are used in too strong a concentration or for too long an exposure. However, in the one experiment we tried, we were not able to obtain any initiation of cell division. In this experiment the eggs were exposed to 1% and to 0.5% Nitromin for periods of 1, 2, 5, 10, 20, 30 and 60 minutes. Our failure to obtain initiation of cell division with the Nitromin solutions was not surprising, for although in every case when egg cells are stimulated to divide, the viscosity of the protoplasm in the interior of the cell is markedly increased, the reverse is not true; for an agent which tends to gel or clot the protoplasm may be too toxic to permit cell division to proceed. Thus in the work on Nitromin, there is only a partial explanation of the Haddow paradox. For though it is true that Nitromin can produce opposite effects on the protoplasmic colloid, we know only that it can suppress cell division and not that it can initiate it.

TABLE V

Effect of ethyl urethane on unfertilized Chaetopterus eggs

Time of exposure to 3% urethane	Viscosity
20 min.	11
32	13
54	13
Control (untreated)	8

TABLE VI

Effect of 3% ethyl urethane in initiating cell division of unfertilized Chaetopterus eggs

Exposure time in minutes	Exp. A: % cleavage after 3½ hours	Exp. B: % cleavage after 6 hours	Exp. C: % cleavage after 8 hours
5	0	0	3
10	0	16	24
15	0	16	50
20	15	40	55
25	26	80	50
30	37	88	45
35	26		29
40	20		34
45	11		20
50	13		23
55	10		22
60	4		6
Control	0	0.05	9

We thought therefore to try the effect of urethane; for this substance, which is known to act both as a carcinogen and as a chemotherapeutic agent for tumors, is presumably less toxic than Nitromin. In relatively weak concentrations, ethyl urethane suppresses cell division in the *Chaetopterus* egg. This is shown in Table IV. This table also shows that in concentrations which suppress cell division, the urethane keeps the protoplasm fluid and prevents the mitotic gelation.

Higher concentrations of urethane have quite the opposite effect. Thus when unfertilized eggs are placed in a 3% solution of urethane, the protoplasmic viscosity increases sharply, as is shown in Table V.

Moreover exposure to 3% urethane can, in a high percentage of cases, cause the egg cells to divide. We have done some experiments of our own to show this, but experiments done by Mr. Herbert Schuel are more complete than ours and we prefer to present them. They are shown in Table VI.

DISCUSSION

Clearly, then, one and the same agent in different concentrations can cause either initiation of cell division or suppression of cell division and these opposite effects are readily correlated with the action the reagent has on the colloidal state of the protoplasm in the interior of the cell.

The facts as we have reported them are so clear cut that they scarcely require additional comment. Therapeutic agents commonly used in the treatment of cancer can prevent cell division by keeping the protoplasm fluid. Some of these agents, when used in different concentrations, can have opposite effects both on the physical state of the protoplasmic colloid and also, in the case of urethane, on the end result. Here, then, we have a way of interpreting the Haddow paradox, and we are able to supply the information Haddow was so concerned about, namely on which part of the cell these agents act and what they do.

But the question immediately arises as to the mechanism of the paradoxical action. In the case of urethane, the answer is rather obvious in the light of what we know concerning the way various anesthetic or narcotic agents act on protoplasm. This subject is discussed at some length by Heilbrunn (1956). Suffice to say here that these agents liquefy the cortical protoplasm and release calcium from it; the calcium thus released enters the interior of the cell and there causes a clotting reaction, which may lead either to excitation or to a complete vacuolization of the protoplasm and death of the cell. But fat solvent anesthetics not only tend to free calcium from the cortex, they also tend to prevent calcium in the cell interior from causing a clotting reaction. There is a large and growing body of evidence in support of these statements (see Heilbrunn, 1956). In addition it should perhaps be noted that ether, which in low concentrations keeps the protoplasm of sea urchin eggs fluid (Heilbrunn, 1920, 1925), can in higher concentrations induce cell division (Mathews, 1900; McClendon, 1910); in these higher concentrations it causes a clotting reaction in the protoplasm.

It is to be hoped that other investigators will join with us in approaching the problem of cancer chemotherapy from the standpoint of the colloid chemistry of protoplasm. At the present time there are so many excellent and well trained workers interested in the metabolic approach, and scarcely anyone concerned with the reasons why the protoplasmic colloid changes in such a way as to form a mitotic spindle. And yet, without mitosis there can be no cancer, and if there are relatively non-toxic ways of preventing mitosis, certainly this is a field that should be investigated on a large scale. Insofar as we know at present, neither the initiation nor the suppression of mitosis depends on any particular metabolic pathway and it certainly does depend on colloidal changes in the protoplasm. Antimitotic substances such as can be extracted from ovaries can indeed be used to cure mice inoculated with a lethal ascites tumor (Heilbrunn, Wilson, Tosteson, Davidson and Rutman, 1957) and in their therapeutic action on this tumor they are at least as effective as nitrogen mustard or Nitromin. Indeed more recent experiments have shown them to be decidedly more effective.

SUMMARY

1. Nitrogen mustard, Nitromin, 6-mercaptopurine, and urethane suppress cell division in *Chaetopterus* eggs.

2. This inhibition of mitosis is due to the fact that these agents keep the protoplasm fluid and prevent the mitotic gelation.

3. In relatively high concentration, both Nitromin and urethane cause a gelation of the protoplasm and in these concentrations, urethane can initiate cell division in a high percentage of the eggs.

4. An interpretation is given of this paradoxical action of reagents in causing either liquefaction or gelation, either suppression or initiation of cell division.

5. The results are believed to provide an explanation of the Haddow paradox.

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FURTHER STUDIES IN THE BEHAVIOR OF COMMENSAL POLYCHAETES

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To date a number of studies have been made on the response specificity of commensal polychaetes (Davenport, 1950, 1953a, 1953b; Davenport and Hickok, 1951; Bartel and Davenport, 1956). In these studies a number of techniques to discern the presence of chemical responses to host have been employed. The subject of specificity and behavior in animal partnerships has recently been reviewed (Davenport, 1955).

During the summer of 1956 further investigations of the behavior of a number of polychaete commensals were conducted at the Friday Harbor Laboratories of the University of Washington. The authors wish to express their appreciation to the Director and staff of the Laboratories for their continued interest and support in these researches. The studies are currently continuing in the Marine Biological Laboratory of Santa Barbara College and have been supported since 1955 by a contract from the Office of Naval Research.

The preliminary investigations cited above had indicated the necessity to compare the behavior of populations of single species of facultative and obligate commensals of diverse host-habit, and to determine whether these populations showed different response specificity. The following studies were directed to that end.

THE FACULTATIVE COMMENSAL PODARKE PUGETTENSIS JOHNSON

Material

The hesionid polychaete *Podarke pugettensis* provides a most interesting subject for behavioral studies. The worm is a facultative commensal, and there appears to be no discernible morphological difference between free-living and commensal members of the species. In the free state the species occurs in great numbers under certain conditions; one may at times collect as many as 15–20 per square yard on the mudflats of Garrison Bay, San Juan Island, Washington. In the Southern California region it may be collected as it settles out of the plankton by suspending open-mouth jars under floats in San Pedro Harbor (D. J. Reish, personal communication), while numbers of adults may be taken by scraping the under surface of floats in the same locality and in Santa Barbara Harbor. It may also be collected by removing large pieces of the growth from pilings, where it occurs near the wood surface deep among the shells of the gastropod *Aletes* and the pelecypod *Chama*. It occurs among the byssus threads of *Mytilus* on pilings. Under these conditions the worms do not appear to be associated with any particular organism, but they certainly seem to thrive in environments of extremely rich organic content. Free-living individuals will be found sporadically in many sorts of environments, particularly where there is rich mud, in the inter-tidal and subtidal. During the summer of 1956 ripe swarming adults were taken at the night light at Friday Harbor for the first time; whether these had been free-living or had come from hosts could not be determined. Swarming has never been observed by us in Southern California.

In California, these worms are commonly associated with the web-star *Patiria miniata* (Brandt), on one individual of which as many as 15–20 may occur. In the Pacific Northwest they are equally common on the mud-star *Luidia foliolata* Grube. There may be considerable variation in the size of worms on both hosts, indicating repetitive colonization by different age classes. In the Puget Sound-Vancouver Island region they may occur on the cushion-star *Pteraster tesselatus* Ives and together with *Nereis cyclurus* Harrington commensal with hermit crabs (Berkeley and Berkeley, 1948). Steinbeck and Ricketts (1941) list the species as commensal with the starfish *Oreaster occidentalis* Verrill in the Gulf of California. That it may occur occasionally with *Pisaster ochraceus* (Brandt) is indicated by a single specimen in the collection of Dr. Olga Hartman, taken by Dr. S. F. Light at Dillon Beach, California. In spite of examining numerous specimens of the common starfish, from Puget Sound to Southern California, we have never found commensal polychaetes of any sort associated with it; there would appear to be a likelihood that the above case was fortuitous.

Method of investigating responses

A.

A choice-apparatus has been designed for the investigation of the possible role of chemical attractants in the regulation of partnerships, such as that between *Podarke* and *Patiria,* in which we have been unable to demonstrate in the individual commensal partner any sharply defined, objectively recordable response to the host (Bartel and Davenport, 1956). The apparatus consists of an aquarium with a central chamber surrounded by and connected by passages with six radially arranged chambers. It may be constructed out of latex as described in the above citation. Our use of the apparatus was as described except that a cover of plywood was added to reduce the possible effects of light. The latter factor was eliminated from consideration in any series of tests by the random selection of test chambers from the possible six. In all the experiments using this apparatus described below, the presence or absence of an attractant factor in one of the chambers among the radial six (the "critical" chamber) is indicated when probabilities, using the null hypothesis that distribution into the six chambers is the result of chance, indicate that either a significant or insignificant number of worms have moved from the central chamber into the critical chamber. Tests averaged from 8 to 12 hours.

Between each test in a series in any experiment the apparatus was washed. Host animals were generally housed during tests directly in one of the radial chambers, but in certain tests indicated below, when host animals were very large, they were housed in a clean, redwood and glass aquarium and the water therefrom siphoned into a radial chamber of the choice-apparatus.

B.

Prior to employing the above described choice-apparatus, Bartel and Davenport (1956) had found, by the simple expedient of placing in dishes large numbers of free-living and commensal *Podarke* together with *Patiria,* that toward this host there is a marked difference in behavior in the two populations; the commensals gathered on the star while none of the free-living worms did. It seemed wise to repeat this experiment in Puget Sound, using both the free-living worms and those commensal with the common host of that region, *Luidia foliolata*. As an experimental animal in behavior studies this starfish presents difficulties; it readily autotomizes its arms when handled or placed in a confined space and hence is not well suited to the latex choice-apparatus. At the same time it is so large that one cannot readily place it in a dish or tray with commensals. We therefore employed a large cement water table (internal dimensions 3′ × 5′ × 3″) in which the starfish could wander freely and "pick up" commensals or in which one could confine the star to a limited space so that the commensals had to "find" it (Fig. 1). In order to so confine the starfish we simply placed a plywood "T" in the table as shown, which would allow free movement of water or worms under its parts but which would trap the star in one corner. Water was introduced at a very slow flow in one corner and drained out at the point shown. One introduced experimental worms at random at the lower end of the table.

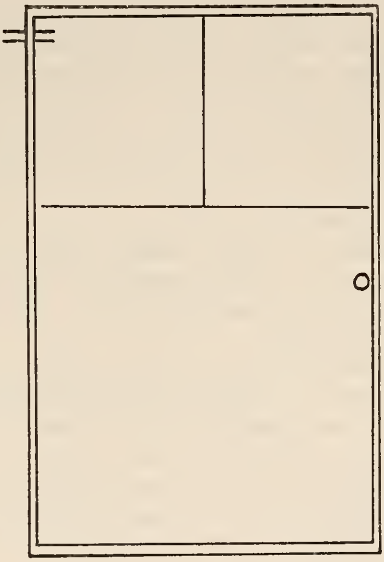

FIGURE 1. Plan of water table.

This apparatus lent itself well to the study of the specificity of response in partnerships in which evidence for a chemical attraction effective at a considerable distance from the host had already been presented (*Arctonoë-Evasterias*, etc.—Davenport, 1950), and also made it possible to conduct tests concurrently with those using the latex apparatus, likewise testing the responses of a large sample of worms in a single test run.

Experiments

Experiment No. 1. Will commensal worms gather on the host *Luidia* when both have the freedom of the water table?

After a time duration of approximately eight hours, 23 out of 36 introduced worms (59%) had moved onto the starfish.

Experiment No. 2. Will commensals, introduced at random at the lower end of the table, find the host if it is trapped at the opposite end?

After a time duration of approximately nine hours, out of 23 worms introduced 13 had found the starfish (56%).

Experiment No. 3. Will free-living *Podarke* (Garrison Bay) gather on *Luidia* when both have the freedom of the water table?

After a time duration of approximately eight hours, out of 32 worms introduced *none* had moved onto the starfish.

Experiment No. 4. Can an attraction for commensal worms be demonstrated in the latex choice-apparatus if water is siphoned from a large aquarium containing the host *Luidia* into one of the six radial chambers?

Six *Luidia* were placed in a large redwood aquarium and three tests followed a control. In the control test with no starfish water in the system, 18 out of 23 worms made a choice and the distribution in the radial chambers was random. In the first test to starfish water, out of 25 worms introduced, 23 made a choice and of the 23, 14 entered the critical chamber ($P < .001$). In the second test all of 20 worms made a choice and of these 8 entered the critical chamber ($P < .01$). In the final test out of 25 worms 11 made a choice, of which 9 entered the critical chamber ($P < .001$).

It is clear that an attraction can be demonstrated with the *Podarke-Luidia* partnership in the latex apparatus. The above data compare very well with those obtained by Bartel and Davenport (1956) with the *Podarke-Patiria* partnership in California, in which two tests gave probabilities of $< .001$ (24 out of 68 and 23 out of 53 entering the critical chamber).

Experiment No. 5. Are free-living worms attracted to a radial chamber into which water from an aquarium containing *Luidia* is siphoned?

In a control test with no starfish water in the system, 16 out of 22 worms made a choice and the distribution was random. In four tests in which 15 out of 25, 16 out of 20, 17 out of 20 and 17 out of 24 made a choice when starfish water was in one of the chambers, the distribution was still purely random. In a single test against starfish water when 16 out of 24 worms made a choice, 8 entered the critical chamber ($P < .01$).

Since the above results were not consistent, further tests were indicated to determine whether or not the release of metabolites in test chambers may occasionally cause free-living worms to distribute themselves unequally in the choice-apparatus, in spite of the fact that under conditions more nearly approaching natural ones, they do not gather on *Luidia* (Experiment No. 3 above).

Experiment No. 6. Can closer propinquity to starfish (and therefore possibly greater concentration of metabolites) perhaps be the answer to the unequal distribution that may occur when free-living worms are tested in the choice-apparatus against *Luidia?* With considerable difficulty a single small *Luidia* was obtained for testing and one test completed with the starfish directly in one of the radial chambers before it autotomized its arms. In this test when 23 out of 30 worms made a choice, 8 entered the critical chamber ($P < .01$).

Experiment No. 7. A further series of tests were conducted at a later date to see whether free-living worms from a different environment than those used in Experiments 5 and 6 might distribute themselves in a non-random fashion when a host was in the system. Free-living *Podarke* from harbor floats in Santa Barbara were tested against the host starfish of California, *Patiria miniata*. In four out of five tests of this kind the distribution was random, but in one, when 19 out of 27 worms made a choice, 10 entered the critical chamber ($P < .001$).

Experiments 5, 6 and 7 have all given an indication that under certain conditions the behavior of free-living worms may be so affected by the presence of a host starfish in the system (perhaps by some metabolite) that their distribution will be non-random. However, they certainly do not respond positively as consistently as their commensal relatives.

Experiment No. 8. It has been demonstrated that commensal *Podarke* show a positive response to the host *Luidia* (Experiments 1, 2, and 4). Will worms from *Luidia* respond to the alternate host *Pteraster tesselatus?*

In two tests in which water from a redwood aquarium containing a single large *Pteraster* was siphoned into one of the six chambers, samples of 19 and 20 worms distributed themselves in a random fashion. But when a smaller *Pteraster* was placed in a radial chamber directly, in one test when 18 out of 23 worms made a choice, 9 entered the critical chamber ($P < .01$) and in the second test when 31 out of 36 worms made a choice, 16 entered the critical chamber ($P < .001$).

Here again propinquity may be a factor, and perhaps the great secretion of mucus produced by handling this starfish may have been a factor in preventing a response in the first two tests, when the starfish was at a greater distance.

Experiment No. 9. How specific is the response of commensals from *Luidia* in the choice-apparatus? Will the commensals respond to non-host starfish?

In a test against *Mediaster aequalis* Stimpson, when 24 out of 31 worms made a choice, 9 entered the chamber containing the host ($P < .01$). In a test against *Pisaster ochraceus* (Brandt) when 9 out of 19 worms made a choice, 5 entered the critical chamber ($P < 1.0$) and in a test against *Evasterias troschelii* (Stimpson) when 17 out of 27 worms made a choice, 11 entered the critical chamber ($P < .001$).

Apparently no response specificity can be demonstrated in the latex choice apparatus when one tests *Podarke* commensal with *Luidia* in Puget Sound.

Experiment No. 10. Do California *Podarke* commensal with *Patiria* show a similar non-specific response in the latex choice-apparatus?

In a series of six control tests against the host alternated with tests against non-host stars, distributions giving probabilities of $< .001$ were obtained in five, while in one test the distribution was random. In the series of 15 tests against *P. ochraceus* (Brandt), *P. giganteus* (Stimpson), *Pycnopodia helianthoides* (Brandt) and *Dermasterias imbricata* (Grube), when samples of from 10 to 44 commensals were used in a single test, all but one test gave completely random distributions. In one test against *P. giganteus* 19 out of 33 worms making a choice entered the critical chamber ($P < .001$).

There would appear to be a marked difference in the response specificity demonstrable in the choice apparatus between worms commensal with *Luidia* in Puget Sound and worms commensal with *Patiria* in California, the latter demonstrating a greater specificity.

The Obligate Commensal, Arctonoë fragilis (Baird)

Material and methods

The polynoid commensal *Arctonoë fragilis* has been studied previously by the authors (citations above). In the first experiments in which it was demonstrated that a marine commensal would respond positively to sea water which housed its host, a Y-tube olfactometer was used, but no detailed studies of response-specificity were made. Such apparatus does not lend itself readily to investigations of specificity of response, since large samples of commensals cannot be tested at once. As it had already been demonstrated that these worms showed an "overt" response to sea water from their host even at a distance, there appeared to be no advantage in employing the latex choice-apparatus. The use of similar water-table tests as described above (Fig. 1) was in order.

A. fragilis has been listed as commensal by Pettibone (1953) with the following asteroid hosts: *Evasterias troschelii; Leptasterias aequalis* and *L. hexactis; Orthasterias koehleri; Pisaster ochraceus; Solaster dawsoni* and *Stylasterias foreri.* We collected and used in the experiments below a large number of worms commensal with *Evasterias,* a few with *Orthasterias* and one (?) with *Solaster dawsoni.* It is unfortunate that cross-specificity studies are made difficult by the fact that it is almost impossible to collect a working sample of commensals from any other host than *Evasterias.* The Berkeleys tell us that at Nanaimo large numbers of *Orthasterias koehleri* can be collected in the inter-tidal zone in winter and early spring; in summer they can only rarely be so collected. Our few specimens of *Orthasterias* were taken in dredges and with the aqualung. It may in fact be possible in the future to compare the behavior of populations of *A. fragilis* from *Evasterias* and *Orthasterias,* by conducting winter experiments. The value of making a thorough comparison of the behavior of two or more separate populations of a single commensal species which inhabits several hosts is obvious. The brief preliminary tests presented below give evidence that the results of such experiments would be most interesting.

Experiment No. 11. Prior to running cross-specificity tests with the two populations of *Arctonoë* available, it was necessary to run a control experiment to determine whether under the conditions of the water table, *Arctonoë fragilis* (commensal with *Evasterias*) would show a response to non-host stars. In four control tests against *Evasterias,* run in alternation with tests against non-host stars, fifteen worms were used in three and fourteen in one. Tests had a duration of not less than nine hours. In the first three 12 out of 15 (79.9%), 10 out of 15 (66.6%), and 10 out of 15 "found" the "trapped" host. In the one test using 14 worms, 13 "found" the host (92.8%). In single tests using fifteen worms against *Pisaster ochraceus, Luidia foliolata, Mediaster aequalis, Hippasteria spinosa* and *Dermasterias imbricata* no worms "found" or moved onto the "trapped" non-host.

Commensal *Arctonoë,* therefore, demonstrate a rather precise response specificity in the water-table.

Experiment No. 12. It appears that commensals from *Evasterias* do not, as one might expect, demonstrate in the water-table a response to stars with which the species is not associated, but what sort of behavior would the worms show in relation to alternate hosts? Will the worms, regardless of host habit, respond to alternate hosts?

To answer this question we presented mixed samples of *Arctonoë,* some from *Evasterias* and some from *Orthasterias koehleri,* with opportunities to "find" each host in the water table. Will each host "sort out" the correct commensals? Unfortunately, because of the above-mentioned difficulty of finding *Orthasterias* we were able to collect only two specimens of *Arctonoë* from this host. In the following experiments these worms were lightly stained in indulin in order to distinguish them from the sample collected from *Evasterias.*

In a series of three water-table tests of this mixed population against *Evasterias* a total sample of 16 worms were used in each. In the first two tests 15 *Evasterias* commensals "ran against" one *Orthasterias* commensal. At the end of nine hours in both tests 10 (75%) of the *Evasterias* commensals had "found" their host while the single *Orthasterias* commensal was still wandering free. In the third test 14 *Evasterias* commensals were "run against" two *Orthasterias* commensals. At the end of nine hours all but one of the *Evasterias* commensals had "found" the host while the two *Orthasterias* commensals were still wandering free.

In two tests against *Orthasterias* a mixed sample of 15 worms was used in the first and of 17 worms in the second. In the first, out of 14 worms from *Evasterias* 13 were still wandering free after nine hours, while the single *Orthasterias* worm had "found" its host. In the second test at the end of the same time, out of 15 *Evasterias* worms none had moved onto the *Orthasterias,* while of the two *Orthasterias* worms, one had "found" the host.

These preliminary experiments against alternate hosts were conducted with a much smaller sample of worms than one would desire and it is hoped that at some time such tests can be repeated with balanced samples. But the tests give an indication of what may be a significant fact. There may, if such responses are not conditioned during development, be good physiological or behavioral races inhabiting different hosts within single commensal polychaete species. That this may be the case was further indicated by a brief experiment in which we tested a mixed population from *Evasterias* and *Solaster.* Accurate identification of the three species of *Solaster* with their two commensal species of *Arctonoë* (*S. stimpsoni* and *S. endeca* with *A. vittata* and *S. dawsoni* with *A. fragilis*), may be difficult. This is particularly true in the case of the worms, in which two species inhabiting closely related hosts may resemble each other greatly; identification can at times only be made by dissection which renders the animals useless for behavior experiments. However, we believe our identification of a single *A. fragilis* on *S. dawsoni* to be correct. When a mixed population consisting of 16 *A. fragilis,* one from *Solaster* and 15 from *Evasterias,* were tested in the water-table against *Solaster,* not one of the *Evasterias* worms moved to the star and yet the single *Solaster* commensal quickly "found" its host.

The Obligate Commensal Arctonoë vittata (Grube)

Material and methods

The polynoid *Arctonoë vittata,* closely related to *A. fragilis,* has perhaps the most interesting variation in host-habit of all the members of the genus. It colonizes certain asteroids, amphineurans, gastropods and polychaetes and within these groups shows a rather precise specificity (Pettibone, 1953). For this reason one might suppose that there could hardly be a commensal better suited to studies of response specificity. Unfortunately, however, as with *Arctonoë fragilis,* it is very difficult to obtain large enough numbers of commensals from each host to make good studies of cross-specificity. In addition to the difficulty in collecting diverse populations of this commensal, one faces the problem of the general inactivity of the worm, which makes studies using a Y-tube or an open water-table tedious in the extreme. It was found, however, that a sample of animals distributed themselves well overnight in the latex choice-apparatus. The following questions were asked and to a certain extent answered, using a single population of worms from the key-hole limpet *Diadora* in the latex apparatus, according to the technique described above.

Experiment No. 13. Will commensals from *Diadora* show a response to the host in the choice-apparatus?

In eight tests samples of from 11 to 29 worms were tested against a group of six limpets in a radial chamber. In two of the eight tests the worms distributed themselves in a random fashion but in six of the tests enough worms chose the critical chamber to give probabilities of $< .001$, $< .01$, < 1.0, < 1.0, $< .001$, and $< .1$.

Experiment No. 14. Do worms commensal with *Diadora* show a response to alternate hosts? Sample alternate hosts tested were the starfish *Luidia foliolata* Grube, *Solaster stimpsoni* Verrill, and *Dermasterias imbricata* Grube; the chiton *Cryptochiton stelleri* Middendorf; and the gastropods *Acmaea mitra* Eschscholtz and *Fusitriton oregonense* (Redfield). Large hosts (*Luidia, Solaster, Dermasterias, Cryptochiton*) were housed in a clean redwood aquarium and the water siphoned from this into one of the radial chambers of the choice-apparatus. Small hosts (*Acmaea, Fusitriton,* small *Cryptochiton* and *Dermasterias*) were placed directly in a radial chamber.

In thirteen tests against these alternate hosts using samples in each of from 13 to 27 commensals the worms distributed themselves in the radial chambers in a random fashion. In a single test of the three against *Fusitriton,* when 20 worms made a choice, 8 entered the critical chamber ($P < .1$), while in the other two tests the distribution, although in both cases the greatest number of worms making a choice entered the critical chamber, gave probabilities $> .1$.

This series of tests indicates that the population of worms commensal with *Diadora* shows under these experimental conditions a rather precise response specificity. Unfortunately, the time duration of this experiment precluded our going further than analyzing the response to an array of available hosts. Certainly, a longer series of tests should be made against *Fusitriton* to determine whether toward this animal, which in some places occupies the identical environment from which the host *Diadora* may be collected, the worms show a constant response.

Discussion

Since the initiation of the study of the specificity of response of polychaete commensals in the summer of 1949, a number of different forms have been investigated. It has been our continued aim to try to correlate this *response* specificity with the known *host* specificity of the species or races. In our effort to make comparisons we have been continually faced with difficulties, some of which have been insurmountable. Among these is the fact that it is extremely difficult to collect large enough samples of worms for such studies in those most interesting species which show within themselves a diversity of host habit; in most such species the worms will, in one locality, occur commonly on one host but very rarely on others. A difficulty encountered in making comparisons between the behavior of different species has been that, as one might expect, not all species exhibit the same sort of response, some showing as individuals a marked or overt response to factors from the host coming from a distance and others merely "accumulating" on or near the host after a passage of time. With such forms as the latter it has been necessary to design special techniques quite different from those used in studies on the former to discern whether or not there is in actuality any response to chemical factors coming from the host. The use of entirely different techniques has made a comparison of results difficult. Some differences in response specificity may turn out to be more apparent than real when some technique has been developed which lends itself equally well to the study of the responses of forms which appear to differ in behavior. Recently we have begun an analysis of the behavior of individual polychaete commensals when under the influence of host factor, using apparatus which may give us some truly comparable data even when studying animals of greatly differing activity or sensitivity.

However, it may be possible at this time, in spite of the above-mentioned difficulties, to make some brief general observations on response specificity in commensal polychaetes.

There would appear to be different categories of response specificity. There is a range of behavior, from the sort which is exhibited by species or populations within species that respond to their host alone, to the sort in which the commensals appear to have no chemical discernment and respond, at least under experimental conditions, to many non-host animals. Specificity of host habit is by no means an indication of specificity of response in experimental apparatus. As an example of the first category which exhibits precise response we have *Arctonoë fragilis* and its behavior in relation to *Evasterias, Orthasterias* and *Solaster*. But there are also species in which populations from one host may give a similar precise response to some, but not necessarily all, alternate hosts, regardless of the absence of any taxonomic affinity between the hosts to which they do respond (*Harmothoë lunulata* from the brittle-star *Acrocnida brachiata* vs. its host and the alternate eunicid *Lycidice ninctta* —Davenport, 1953b). Among such species of diverse host habit there may be a population occurring on one of the array of hosts which responds to its host alone, in spite of the fact that other populations of the same species respond to several alternates (*Harmothoë lunulata* from *Leptosynapta inhaerens*). A further category consists of those species which respond with the same intensity to the known alternate hosts but with reduced intensity to a number of non-host relatives of their hosts (*Acholoë astericola* from *Astropecten irregularis* vs. its host and the alternate *Luidia ciliaris*, as well as non-host stars—Davenport, 1953a). Finally we have a category which, though somewhat unexplainable, can be demonstrated to exist even when using a standard technique. In some facultative commensals there appear to be populations (*Podarke* on *Patiria*) which show a precise response specificity to their host alone and others (*Podarke* on *Luidia*) which seem unable to discern the difference between their host and other non-host animals.

It is therefore quite apparent that it is pressing to determine, particularly in forms such as *Podarke*, whether responses are inherited or conditioned. Although it would seem difficult to imagine a mechanism whereby such a host response could be conditioned in forms such as *Podarke*, the early stages of which (in the laboratory) remain in the plankton for some 30 days, nevertheless only successful breeding and settling experiments will give us the answer.

SUMMARY

1. A new water-table test apparatus for the investigation of commensal response behavior is described.

2. Evidence is presented that the two populations of the facultative commensal *Podarke pugettensis* (Polychaeta: Hesionidae) which may be termed "commensal" and "free-living" differ markedly in their response to host animals, the commensal worms showing a strong tendency to respond positively to the host and the free-living worms not doing so.

3. Commensals with the starfish *Luidia* in Puget Sound appear to respond with almost equal intensity to other non-host animals (the response is not specific), while commensals of the star *Patiria* in Southern California show a more precise and specific response. This behavioral difference remains unexplained.

4. The behavior of three populations of the obligate commensal *Arctonoë fragilis* (Polychaeta: Polynoidae) was compared. Evidence is presented that each population (one commensal with the star *Evasterias*, one with the star *Orthasterias* and one with the star *Solaster*) shows a response to its host alone.

5. The response behavior of *Arctonoë vittata* (Polychaeta: Polynoidae), an obligate commensal of diverse habit, was investigated in relation to a number of its alternate hosts.

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ON THE MORPHOLOGY OF THE NEPHRIDIUM OF NEREIS VEXILLOSA GRUBE[1]

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It has been well established that certain of the Nereidae are capable of surviving in waters of low salinity. In the field this is demonstrated by their invasion of brackish and even fresh waters (Johnson, 1903; Hartman, 1938; Smith, 1950, 1953, 1956), and has also been suggested in the experimental work of some investigators (Schlieper, 1929; Nomura, 1930; Jürgens, 1935; Sayles, 1935; Beadle, 1937; Ellis, 1937; Topping and Fuller, 1942; Krishnan, 1952; Smith, 1955). In spite of the fact that many physiological studies have been carried out on various nereids, only a few morphological descriptions of the presumed osmoregulatory organ of these annelids, the nephridium, are to be found in the literature.

The first detailed description of nephridial morphology was made by Goodrich (1893) on *Nereis diversicolor.* He found three sections along the length of the nephridial tubule, each grading into the next. The sections varied in respect to the presence or absence of cilia, the diameter of the tubule lumen, and the extent of convolution of the tubule.

Fage (1906) studied *Perinereis cultrifera,* confining his work to living material. He also found areas of ciliation, but these differed from those observed by Goodrich in *N. diversicolor.* Much later, in his extensive review of observations of nephridia and genital ducts, Goodrich (1945) re-stated his earlier findings but added little to them. In his work on *Lycastis indica, Nereis chilkaensis,* and *Perinereis nuntia,* Krishnan (1952) made a study of the nephridia of each species and compared them with respect to vascularization and size, relative to body size.

Because of the paucity of adequate morphological studies on nereid nephridia, it was felt that further study was in order, to provide a better basis for physiological work, and for later studies of comparative functional morphology.

MATERIALS AND METHODS

Specimens of *Nereis vexillosa* utilized in this study were obtained from a breakwater at the Berkeley Yacht Harbor, in San Francisco Bay, California. Although no salinity determinations were made at this time, the annual salinity range for this area is from 26.3 to 32.4‰ (approximately 73 to 90% sea water), according to Sumner *et al.* (1914) and Miller *et al.* (1928).

The worms were relaxed by gradual addition of 30% ethyl alcohol, fixed in Bouin's fixative and serially sectioned at eight micra. They were then stained with

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Harris' haematoxylin and counterstained with eosin Y. Other fixatives and stains were utilized, but these gave poor results.

In order to obtain a graphic representation of the canal as it passed through the nephridial mass, a plaster-of-Paris reconstruction was made. Camera lucida drawings were transferred to sheets of paraffin of proper thickness, and as the replica was built up, the lumen of the canal was hollowed out. Later, the canal was filled with plaster, and the surrounding paraffin was melted away.

NEPHRIDIAL MORPHOLOGY

The nephridia of *Nereis vexillosa* are paired organs in the coelomic cavity of each segment, just lateral to the ventral longitudinal muscle bundles, near the base of each parapodium. Within the broad base of attachment of the nephridium to the body wall, the nephridial canal opens to the exterior by way of the nephridiopore (Figs. 1, 2, 6, NPR). The internal opening of the nephridial canal, the nephrostome (Figs. 1, 3, 5, NST), is found at the end of an anterior extension of the canal (the post-septal canal, Figs. 1, 3, PSC) which leaves the mass of the nephridium and passes anteriorly, through the inter-segmental septum (Figs. 1, 3, 5, SEP), to the next segment.

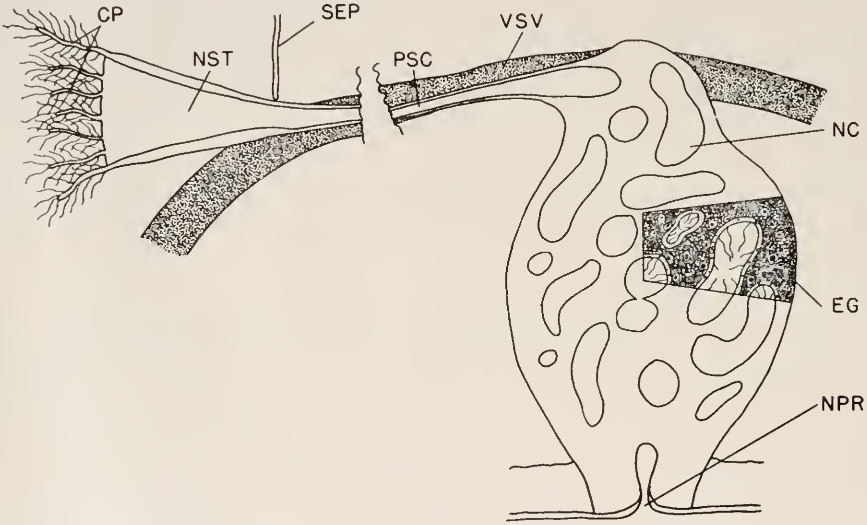


FIGURE 1. Schematic diagram of a nephridium of *Nereis vexillosa*.

Externally, the nephridium is a discrete mass of tissue, varying from globose or pyriform to irregular in shape. The surface may be ridged to some extent, because of the passage of the nephridial canal close to the surface. In mature worms of from 55 to 70 segments (*ca.* 70 mm. long when relaxed) nephridia were approximately 250 micra at their widest and about the same dorso-ventrally. They measured nearly 200 micra through their antero-posterior axis, exclusive of the post-septal canal and nephrostome which, in themselves, were about 300 micra in length.

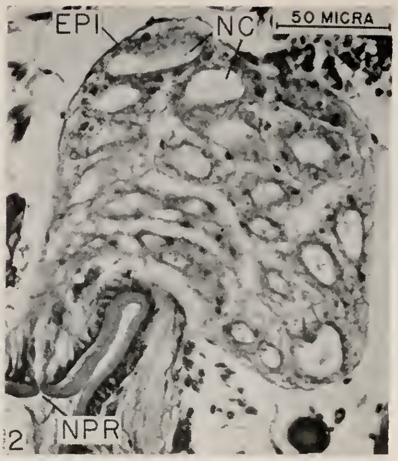
In section, the convoluted nephridial canal is seen as many perforations in the nephridial tissue (Figs. 1, 2, 3, 4, NC). A fairly discrete wall often lines the tubule, although, usually, the boundaries of these cells are difficult to resolve (Fig. 4). The surface of the nephridium is covered by a single, very thin layer of squamous coelomic epithelium cells (Fig. 2, EPI). It is well to point out that, as far as is known, all nereid nephridia have this general configuration, *i.e.,* a convoluted canal in the nephridial mass. An apparent contradiction to this fact occurs in a recent text (Prosser *et al.*, 1950, pp. 17–18) where it is stated that ". . . the nephridium of *N. cultrifera* is a simple sac." This is justified by a figure modified from Jürgens (1935) which had been redrawn from the work of Fage (1906). Fage's original figure was a surface view of the nephridium of *Nereis cultrifera,* and in subsequent copyings, the delicate shadings which showed surface texture were lost, and the figure evolved to that of an optical section of the nephridium. The fate of the figure notwithstanding, Fage (1906, p. 338) described the nephridium as a ". . . masse spongieuse, perforée en tous sens par un grand nombre de canaux"; therefore, it is certainly not a simple sac. The matrix of the nephridium is a highly vaculated, syncitial, network of loose connective tissue, which serves to bind the convolutions of the nephridial canal.

Nuclei are of two types, a smaller kind, rich in chromatic material (3 × 5 μ), and a larger, clearer kind (5 × 10 μ). Nuclei of both types are usually found in or near the canal walls, and only occasionally are they seen isolated in the matrix tissue (Figs. 2, 3, 4).

No blood vessels have been noted within the nephridial mass, and in only two places is the nephridial system approached by vascular elements. One blood vessel passes over the anterior face of the nephridium (Fig. 3, BV) and another, the ventral segmental vessel, lies along the post-septal canal (Figs. 3, 5, VSV). In neither case is the association intimate, and there is little opportunity for the transfer of materials from one structure to the other. Although neither Goodrich (1893) nor Fage (1906) mentions the relationships between the nephridium and the vascular system, Krishnan (1952) has indicated that in *Lycastis indica, Nereis chilkaensis,* and *Perinereis nuntia,* blood vessels are found in close association with nephridia. He also points out that the extent of nephridial vascularization is inversely related to the salinity of the environment.

Occasionally, eosinophilic granules have been seen in the tubule walls and the matrix tissue (Fig. 4, EG). There is no special distribution along the length of the canal, and no special accumulations in the nephridial mass. Goodrich (1893) mentioned minute granules in the cells of the tubule wall in *N. diversicolor* and considered these to be composed of excretory materials. Fage (1906) observed that with the addition of neutral red to the sea water bathing the freshly-dissected nephridia of *Perinereis cultrifera,* granules were formed which were similar to those which were observed in untreated nephridia, and which Fage terms, excretory granules (grains d'excrétion).

The post-septal canal (Fig. 3, PSC) is produced anteriorly as an extension of the nephridial canal, and is covered by the same thin squamous layer which invests the nephridium. Nuclei are uniformly scattered along its length and are concentrated in a band at the level of the septum (Fig. 5, NB). Anterior to the septum, the post-septal canal enlarges and gives rise to the funnel-shaped nephrostome. The lateral margin of the nephrostome is slightly recurved, and around the entire rim, there are numerous cytoplasmic processes (Fig. 5, CP).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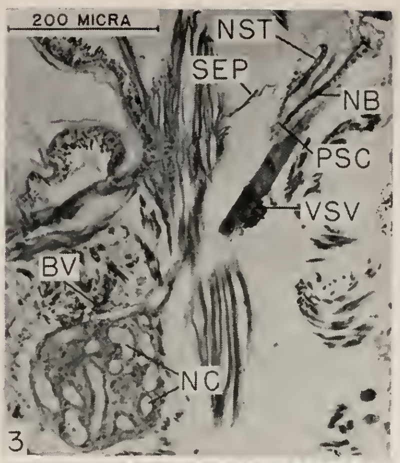
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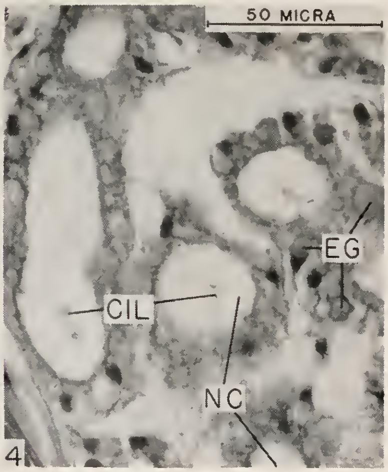
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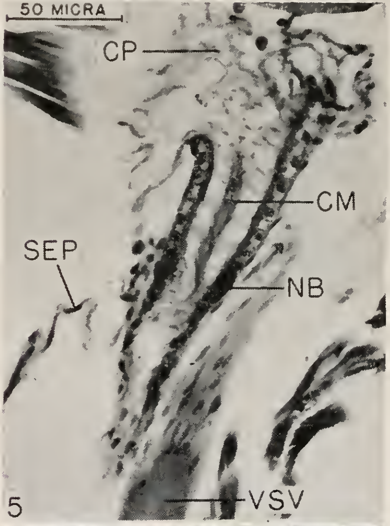
4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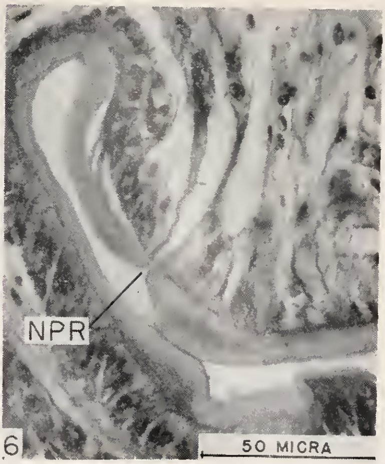
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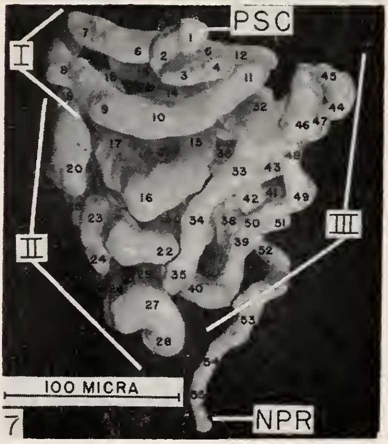
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Figs. 2–7.

At the terminal end of the nephridial canal, the wall of the lumen thins and becomes continuous with the invaginated cuticle of the outer surface to form the nephridiopore (Fig. 6, NPR). In this respect, the structure of this area differs from that of *N. diversicolor* and *N. chilkaensis*, for Goodrich (1893) states that in *N. diversicolor* the wall of the tube pierces the epidermis, and Krishnan (1952) presents a figure showing the same condition in *N. chilkaensis.*

By means of camera lucida drawings, the entire course of the convolutions of the nephridial canal was followed from the nephrostome to the nephridiopore. It was then possible to ascertain the extent of ciliation within the lumen of the canal (Fig. 4, CIL). It was seen that the ciliation of the nephrostome is extremely heavy, and forms a tightly wound swirl in the throat of the nephrostome (Fig. 5, CM). The heavy ciliation is maintained throughout the rest of the post-septal canal, and gives a characteristic "star" or "wagon wheel" aspect to transverse sections of this structure. The ciliation of the portion of the canal included within the nephridial mass is constant, but not uniform. No obvious areas of heavy or sparse ciliation, such as were noted by Goodrich (1893) in *N. diversicolor*, have been observed here, and in general, the midportion is only slightly more heavily ciliated than either end. In the region of the nephridiopore (Fig. 6), the canal is devoid of cilia for about the last 40 micra of its length. It has also been noted that in *N. vexillosa* the cilia are never attached on only one side of the lumen as Goodrich reported in *N. diversicolor.* In addition, no tufts of cilia, such as Fage (1906) has described in the nephridial canal of *Perinereis cultrifera*, have been seen here.

The plaster reconstruction (Fig. 7) shows that after the post-septal canal joins the nephridial mass, the canal is thrown into fairly tight, somewhat spiraled convolutions (I, Fig. 7). It then winds back and forth along the medial surface, roughly parallel to the antero-posterior axis (II, Fig. 7). Next, it passes to the mid-lateral portion of the mass and is once more tightly convoluted (III, Fig. 7). This condition gives way to a relatively straight length which terminates at the nephridiopore (Fig. 7, NPR).

In addition to affording a three-dimensional view of the path of the canal through

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Key to lettering: BV, blood vessel; CIL, cilia; CM, mass of cilia; EG, eosinophilic granules; EPI, coelomic epithelium; NB, band of nuclei of nephrostome; NC, nephridial canal; NPR, nephridiopore; NST, nephrostome; CP, cytoplasmic processes of nephrostome; PSC, post-septal canal; SEP, intersegmental septum; VSV, ventral segmental blood vessel; I, II, III, first, second, and third regions of the nephridial canal, respectively.

FIGURE 2. General view of a nephridium, transverse section (8 μ, Harris' haematoxylin and eosin; the cavity extending internally from the area of the nephridiopore, NPR, is a longitudinal fold of the body wall; the ventral nerve cord is to the left of the figure and the parapodium is to the lower right).

FIGURE 3. View of nephridium and its associated nephrostome, frontal section (8 μ, Harris' haematoxylin and eosin).

FIGURE 4. Detailed view of nephridial tissue (8 μ, Harris' haematoxylin and eosin).

FIGURE 5. Detailed view of the nephrostome of Figure 3.

FIGURE 6. Detailed view of the nephridiopore, transverse section (8 μ, Harris' haematoxylin and eosin; the large cavity extending toward the upper left of the figure is a longitudinal fold of the body wall).

FIGURE 7. Plaster reconstruction of the nephridial canal, view of the anterior face. (The consecutive numbers indicate the course of the canal; section I, 1–18; section II, 19–30; section III, 31–55.)

the nephridium, the reconstruction shows three regions which merge gradually into one another. After the narrow post-septal canal joins the nephridium, the canal becomes slightly enlarged through the first region of convolution (I, Fig. 7). The canal is then further enlarged to its maximal diameter as it passes to the medial surface (II, Fig. 7). It becomes narrowed in the second series of tight convolutions (III, Fig. 7), and it is at its minimal diameter as it passes to the nephridiopore. This condition is reflected to some extent in Figure 8, which is a graph of the inner diameter along the length of the canal (a measurement of the outer diameter, which would show the thickness of the canal wall, was not possible, due to the poor definition of the cells of the wall). It is of interest to mention the regions of the tubule within the nephridium, as determined by Goodrich and Krishnan, although such differences may well be due to observations of different species. In *N. diversicolor,* Goodrich (1893) found a much convoluted portion with few cilia, into which the post-septal canal led. The next region was very narrow, and the cilia here were confined to one side of the canal. The last section was short, less convoluted, moderately wide, and without cilia. In *N. chilkaensis,* Krishnan (1952) found that the first portion of the canal, as it enters the nephridium, is convoluted and ciliated. The next portion is wider, without cilia, and longer than the preceding section. This latter portion gives way to a canal which leaves the body of the nephridium and terminates at the nephridiopore.

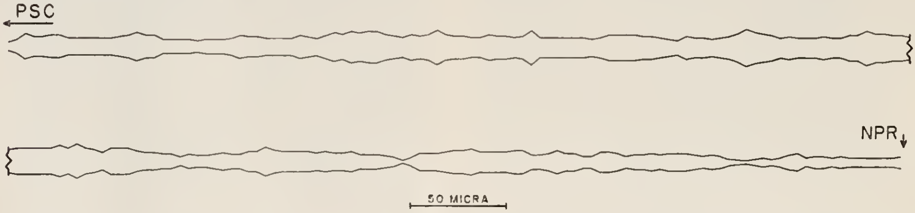

FIGURE 8. Graphic representation of the inner diameter of that part of the nephridial canal within the nephridial mass (reconstructed by measuring the shortest diameter of elliptical sections of the nephridial canal).

The mean diameter of the nephridial canal upon which the reconstruction (Fig. 7) and the graph (Fig. 8) were based, was 24 μ, and the over-all length of the canal within the nephridial mass (not including the nephrostome or the post-septal canal) was approximately 1.7 mm.

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SUMMARY

1. The morphology of the nephridia of *Nereis vexillosa* Grube is described.

2. Comparisons are made with the morphology of the nephridia of certain other nereids and differences are noted. Chief among these are, in *N. vexillosa:*

a. that ciliation extends along the whole length of the nephridial canal, with the exception of a short region just preceding the nephridiopore;

b. that three general regions of the nephridial canal are noted, on the basis of the diameter and the amount of convolution;
c. that the wall of the nephridiopore appears to be inserted on the invaginated surface cuticle.

3. A reconstruction of the nephridial canal is presented in which the course of the canal is readily seen.

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STUDIES ON THE ISOLATED ISLET TISSUE OF FISH.[1] II. THE EFFECT OF ELECTROLYTES AND OTHER FACTORS ON THE OXYGEN UPTAKE OF PANCREATIC ISLET SLICES OF TOADFISH, USING THE CARTESIAN DIVER MICRORESPIROMETER

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We have undertaken a detailed characterization of the metabolism of islet tissue because we believe that these studies may provide the basis for understanding the factors which control insulin synthesis and the mechanism by which alloxan and other toxic agents selectively kill the insulin-producing cells (Lazarow, 1949). In approaching this problem we have found it convenient to use the toadfish as an experimental animal. Whereas in mammals the islet tissue is distributed throughout the pancreas in a million or more individual islets of Langerhans totaling only 1% of the pancreatic mass, in the toadfish the islet cells are segregated into one or more discrete bodies which are located in the mesentery and which are called the principal islets (Diamare, 1899; Rennie, 1905). The pancreatic acinar tissue in the toadfish does not form a definite organ; rather, it is diffusely scattered throughout the mesentery, along the bile ducts, and within the liver.

In a previous study (Lazarow and Cooperstein, 1951) we have measured the activity of certain specific enzymes (cytochrome oxidase and succinic dehydrogenase) in normal toadfish islet tissue homogenates. However, in order to characterize the over-all metabolic pathways in islet tissue, it is important to measure the endogenous oxygen uptake as well as that following the addition of specific exogenous substrates. We have therefore studied the endogenous respiration of islet tissue slices and in the present paper we are reporting the effect of varying pH, tonicity, electrolyte composition, and other factors. By means of these studies we have been able to define the conditions under which maximal respiration of the islet tissue slices occurs. This should provide a base-line for subsequent work, which will include a study of (a) the effect of various substrates known to play a role in intermediary metabolism, (b) the effect of various inhibitors and, (c) the effect of hormones and other agents which influence the blood sugar level and/or insulin secretion.

METHODS

Mature toadfish, *Opsanus tau,* weighing 200 to 600 gm. were used. During the summer months the animals were kept in a running sea water tank, and they were killed within several weeks of the time that they were caught. During the fall and winter months the toadfish were kept in a live car for varying periods of time (1–6 months) after which they were shipped inland by air express from the Marine Biological Laboratory, Woods Hole, Massachusetts. The fish were stored for periods up to one week in an aerated sea water tank (30-gallon crock). The temperature of the sea water was maintained below 20° C. by circulating cold water through the inside of submerged lead coils.

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The cartesian divers used were of the cylindrical type without a bulb and they were silicone-treated prior to use. Before the fish were killed, the divers were filled with the various liquid media to be studied; they were completely filled except for a 1–2 mm. air bubble at the very bottom of the diver. The divers were then cooled to 0° C. by placing them in a cooling block. During the intervals between manipulative procedures, the divers were stored in this cold block. After the toadfish were decapitated, the islets were dissected from the mesentery and placed on a piece of Parafilm. The connective tissue capsule surrounding the islet was removed. A petri dish cover, containing a piece of wet filter paper, was placed over the tissue; this served as a moist chamber. The razor blade used for cutting the islet slices was previously cleaned with sodium hydroxide (to remove all traces of oil), thoroughly rinsed in tap water, and finally washed in distilled water. In general the islet obtained from one toadfish was used for each day's experiment; it was cut into eight pieces, each weighing approximately 0.1 to 0.2 mg. wet weight. A slice of islet tissue was then placed at the lower air-liquid meniscus in each of eight divers with the aid of a fine stainless steel needle. Most of the liquid medium was then removed from the diver using a micro pipette; however, a cylindrical segment of medium, about 1 mm. in length, was left behind to form the tissue seal containing the islet slice. With the aid of the cartesian diver filler (Lazarow, 1950), the neck seals of sodium hydroxide, oil and flotation medium were successively placed, in the stated order, above the tissue sample. The divers were then transferred to a thermostatically controlled water bath maintained at 25° C. ± 0.01° C. The pressure was measured using a Wallace Tiernan gauge (Belleville, New Jersey) which was initially suggested and used by Claff (personal communication, 1948). This gauge was calibrated in millimeters of Brodie's solution. A compensating device (Lazarow and Bloomfield, unpublished) connected to the outer chamber of the gauge was used to minimize the effect of changes in barometric pressure. An initial reading was taken after a 20-minute equilibration period. Subsequent readings were taken at 20-minute intervals during the next hour. Thermobar divers usually showed a pressure change of less than 4 mm. per hour, whereas experimental divers showed a change up to 100 mm. per hour. At the end of the experiment the divers were removed from the water bath and the sodium hydroxide, oil and flotation medium seals were removed. The tissue slice plus the tissue seal were transferred from the diver to a smaller model of the conical-tipped micro homogenizer (Lazarow and Portis, 1951), using a capillary pipette. The tissue was homogenized in 100 μl. of water and three 25-μl. aliquots were removed for protein estimation. The protein was determined by a modification of the method of Lowry, Rosenbrough, Farr and Randall (1951). Twenty-five μl. of the sample were mixed with 250 μl. of the protein reagent; the mixture was allowed to stand at 45° C. for ten minutes and, at the end of this time, 25 μl. of the diluted phenol reagent were added. The absorption was read after 15 minutes at room temperature in the Beckman spectrophotometer at 700 mμ. The amount of protein in the sample was determined by comparing the extinction with that obtained using a standard serum albumin solution. The metabolic activity was expressed as millimicroliters (mμl.) of oxygen taken up per microgram of protein per hour.

Results

At the time this study was begun the osmotic pressure of toadfish blood (sample obtained from the gill) had been determined and found to be equivalent to 0.19 *M* NaCl (Green, personal communication). In the first group of experiments shown in Table I, the respiration of islet tissue in 0.19 *M* NaCl was compared with that observed in a saline-phosphate buffer mixture (containing 0.144 *M* saline and 0.033 *M* Na_2HPO_4–KH_2PO_4 buffer, pH 7.4). In three series of experiments the oxygen uptake of islet tissue in the saline-phosphate mixture was 43 to 153% greater than that observed in 0.19 *M* saline. The *p* values indicated that the differences were all highly significant. Table I also shows that in a group of 11 experiments in which the saline-phosphate mixture was used, there was excellent reproducibility in the average oxygen uptake for the individual series. On the other hand, in the three series of experiments in which 0.19 *M* NaCl was

Table I

Effect of phosphate buffer on islet tissue respiration

Series	mμl. O_2/μg. protein/hr. in saline (0.19 *M* NaCl)			mμl. O_2/μg. protein/hr. in saline-phosphate (0.033 *M* Na_2HPO_4–KH_2PO_4, pH 7.4 + 0.144 *M* NaCl)			p**
	No. determinations	Aver.	σ*	No. determinations	Aver.	σ*	
1	11	1.42	0.80	10	2.24	0.72	.012
2	17	0.78	0.42	14	1.97	0.89	<.001
3	17	1.54	0.41	17	2.20	0.70	.001
4	—	—	—	15	2.62	1.00	—
5	—	—	—	8	2.63	0.84	—
6	—	—	—	9	2.26	1.00	—
7	—	—	—	19	2.08	0.99	—
8	—	—	—	24	2.12	0.95	—
9	—	—	—	16	2.72	0.78	—
10	—	—	—	15	2.32	0.97	—
11	—	—	—	14	2.14	0.79	—
Aver.	45	1.22†	—	161	2.28†	0.37†	

* $\sqrt{\frac{\Sigma\text{ (deviations from mean)}^2}{\mathrm{N}}}$.

** Calculated from the formula $\dfrac{\text{Difference}}{\sqrt{\dfrac{\sigma_1^2}{\mathrm{N}_1}+\dfrac{\sigma_2^2}{\mathrm{N}_2}}}$.

† Each series taken as one figure.

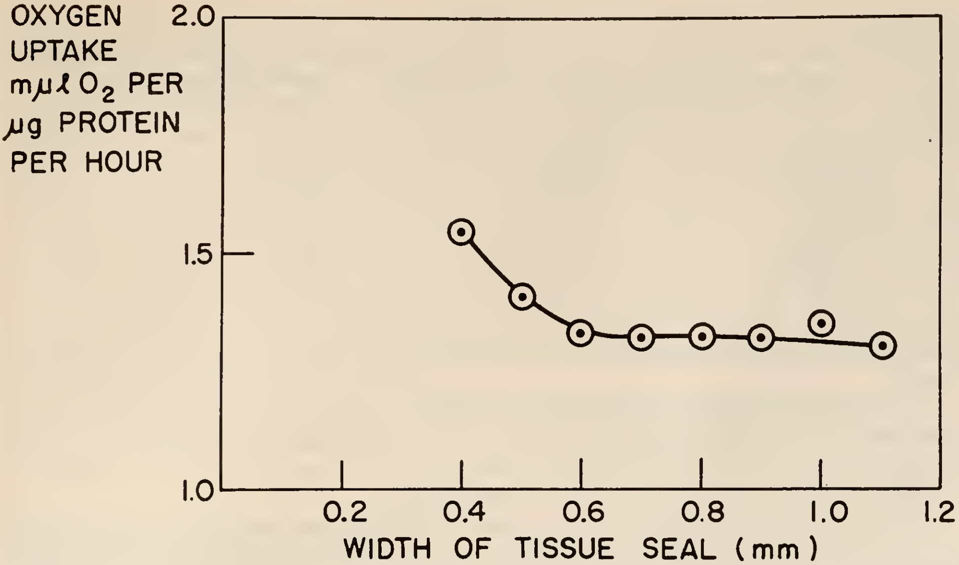

Figure 1. Effect of cartesian diver tissue seal width on the respiration of islet slices suspended in 0.19 *M* NaCl. All of the points on the graph in which the tissue seal width was 0.8 mm. or less represent an average of 19 or more individual determinations; the other points represent the average of 5 to 13 individual determinations.

used, the reproducibility was not as good as that observed in the saline-phosphate mixture. A possible explanation of this is shown in Figure 1. The volume of the tissue seal varied considerably from experiment to experiment and in Figure 1 the oxygen uptake of islet slices in 0.19 *M* saline was plotted against the volume of tissue seal in the diver, *i.e.*, the volume of fluid medium surrounding the tissue slice. The metabolic activity of the islet slices in 0.19 *M* saline decreased as the volume of the tissue seal increased. Moreover, this did not occur when phosphate was used; therefore, this greater metabolic variability in saline may be the result of variable dilution of the phosphate extracted from the tissue. Since phosphate addition stimulated the respiration of islet, one would expect that the oxygen uptake would be greater in the divers with small tissue seals.

The oxygen uptake of the islet tissue slices, when expressed as mμl. of oxygen per

Table II

Effect of different proportions of Na^+ to K^+ on islet tissue respiration. All media contained 0.033 M phosphate buffer pH 7.4 + 0.144 M NaCl or KCl. The proportions of Na^+ to K^+ were changed by varying the buffer and saline cations

Ratio Na^+/K^+ in medium	No. determinations	Aver. mμl. O_2/μg. protein/hr.	σ
100/0	16	2.77	1.26
96.5/3.5*	15	2.62	1.00

* Saline-PO_4 mixture as described in Table I.

Table III

Effect of different proportions of Na^+ *to* K^+ *on islet tissue respiration. All media contained 0.033 M phosphate buffer pH 7.4 + 0.144 M NaCl or KCl. The proportions of* Na^+ *to* K^+ *were changed by varying the buffer and saline cations*

Ratio Na^+/K^+ in medium	No. determinations	Aver. mμl. O_2/μg. protein/hr.	σ
100/0	16	2.77	1.26
80/20	16	3.24	1.46
0/100	17	3.26	1.33

μg. of tissue protein, appears to be independent of the size of islet tissue slice used.

Since the stimulation observed in the saline-phosphate buffer medium could be due either to (a) the addition of potassium ion (contained in the buffer), (b) buffering action, or (c) a specific phosphate ion effect, experiments were carried out in order to determine which factor was responsible.

The effect of varying the potassium and sodium ion concentrations of the medium is shown in Tables II and III. In the usual saline-phosphate buffer medium there are three and one half parts of potassium to ninety-six and one half parts of sodium. When an all-sodium phosphate buffer was used (prepared by mixing sodium monobasic and sodium dibasic phosphates), there was no change in metabolic activity of the islet slices. When higher potassium ion concentrations were used (Table III), the activity was about 20% higher than that observed with an all-sodium medium. However, analysis of these results showed that this difference was not statistically significant. Since the addition of phosphate buffer increased the metabolic activity of islet slices by 43 to 153%, it may be concluded that this stimulation is not due to the addition of the potassium ion contained in the buffer.

The effect of pH. Various saline-phosphate-buffer mixtures were prepared in which the pH of the phosphate buffer was varied. Table IV shows that there were no significant differences in the metabolic activity of islet slices when the pH was varied from 6.2 to 8.0; the *p* values were all greater than 0.3. This finding is in keeping with the studies previously reported by other investigators (Elliott and Birmingham, 1949) who suggest that the internal pH of tissue slices is maintained fairly constant over a wide range of external pH. This finding therefore suggests that the stimulatory effect observed with phosphate buffer addition (Table I) was

Table IV

Effect of pH on islet tissue slice respiration. In all cases the medium consisted of 0.144 M NaCl + 0.033 M Na_2HPO_4–KH_2PO_4 *buffer*

pH of medium	No. of determinations	Aver. mμl. O_2/μg. protein/hr.	σ
6.2	11	2.39	0.67
6.8	14	2.46	0.89
7.4	14	2.14	0.79
8.0	15	2.53	1.25

due to a specific phosphate ion effect rather than to the ability of the phosphate buffer to maintain the pH of the medium.

The effect of buffer type. The metabolic activity of islet slices was studied in phosphate, tris (trishydroxyaminomethane), and veronal buffers at pH 7.4 and the results are shown in Table V. The highest metabolic activity was observed with phosphate buffer. With an equimolar concentration of tris buffer, the oxygen uptake was lower than with phosphate buffer (p value $< .001$), but it was of the same order of magnitude as with 0.19 M saline (Table I). With veronal buffer the metabolic activity was 86% lower than with phosphate and much lower than with 0.19 M saline. Thus veronal buffer inhibits the metabolic activity of islet slices. Since in the presence of 0.033 M tris plus 0.033 M phosphate buffer the O_2 uptake was the same as in phosphate, the low activity in tris buffer was not due to inhibition, but rather to the absence of phosphate.

The effect of phosphate concentration. A large series of experiments were carried out in which varying concentrations of the Na_2HPO_4–KH_2PO_4 buffer (pH 7.4) were added and the tonicity of the medium was maintained equivalent to 0.19 M NaCl by adjusting the NaCl concentration. These results are shown in Figure 2. There was a progressive increase in the oxygen uptake as the phosphate ion concentration was increased. Maximal stimulation of metabolic activity was observed at a phosphate ion concentration of 0.066 M. The stimulation observed in the presence of 0.066 M phosphate was highly significant. A comparison of the activity in 0.066 M phosphate with that observed in the absence of phosphate, or in the presence of 0.002 M or 0.008 M phosphate, gave a p value < 0.001. The difference between 0.066 M and 0.033 M phosphate is also probably significant (p value $= 0.057$). At a phosphate ion concentration greater than 0.066 M there was no further stimulation; in fact the value at 0.136 M is actually 5% lower than at 0.066M.[2] This difference, however, is not significant ($p = 0.6$).

TABLE V

Effect of buffer type on islet tissue slice respiration. In all cases the medium contained 0.033 M buffer, pH 7.4; the medium containing both tris and phosphate buffer was 0.033 M with respect to each. Enough NaCl was added to each medium to maintain a tonicity equivalent to 0.19 M NaCl

Buffer in medium	Series I			Series II		
	No. determinations	Aver. mμl. O_2/μg. protein/hr.	σ	No. determinations	Aver. mμl. O_2/μg. protein/hr.	σ
Na_2HPO_4–KH_2PO_4	15	2.32	0.97	19	2.08	0.99
Tris	14	1.29	0.64	18	1.60	0.47
Veronal	6	0.33	0.13	—	—	—
Tris Na_2HPO_4–KH_2PO_4	—	—	—	19	2.02	0.52

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[2] Later studies on the effect of tonicity showed that at the high concentrations of phosphate one would have expected a large decrease in metabolic activity due to the increasing tonicity of the medium. In the presence of 0.136 M phosphate, this decrease should have been much larger than the 5% actually observed. The fact that a decrease of only 5% was observed using 0.136 M phosphate suggests that the higher concentration of phosphate is effectively stimulating the metabolic activity but that this stimulation is masked by inhibitory effects of increasing

The effect of tonicity. In order to study the effect of tonicity, the oxygen uptake of islet slices was measured in media of varying salt concentrations. However, since the phosphate buffer concentration that gives maximal stimulation (0.066 *M*) has a tonicity equivalent to 0.093 *M* NaCl, it was necessary to reduce the phosphate ion to a suboptimal value if lower tonicities were to be used. Therefore additional studies were carried out with lower phosphate concentrations. Where necessary the tonicity was adjusted by adding NaCl. Table VI shows that the oxygen uptake increased with decreasing tonicity. When the tonicity of the medium was equivalent to 0.093 *M* NaCl, the metabolic activity of islet slices was 60% greater (p = 0.006)

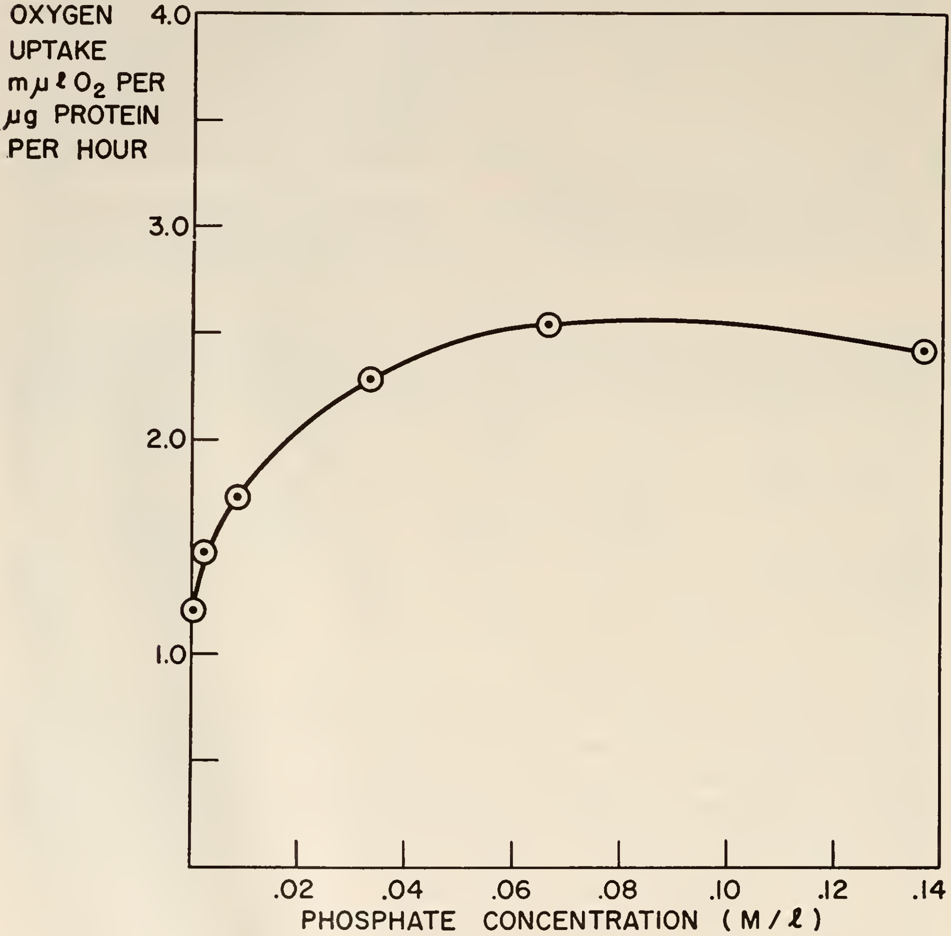

FIGURE 2. Effect of phosphate ion concentration on the respiration of toadfish islet slices. The tonicity of the medium was maintained equivalent to that of 0.19 *M* NaCl; pH = 7.4. Each of the points represents the average of 32 to 161 individual determinations.

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tonicity. Thus the appearance of a maximum at a phosphate concentration of 0.066 *M* may be more apparent than real.

than that observed when the tonicity was equivalent to 0.19 M NaCl. When the tonicity was decreased further, the oxygen uptake diminished. Furthermore it should be noted that at a tonicity equivalent to 0.093 M NaCl, a progressive decrease in the phosphate ion concentration from 0.066 to 0.033 to 0.017 M gave a slight but progressive decrease in metabolic activity of the islet slices. This decrease is of the order of magnitude that would be expected from the phosphate curve (Fig. 2).

In order to explore more fully the effect of the tonicity of the medium, a series of experiments were carried out in which the phosphate ion concentration was maintained at 0.033 M (a slightly sub-optimal phosphate level) and in which the tonicity was varied between a sodium chloride equivalent of 0.048 M and 0.50 M. The results are shown in Figure 3. The maximum activity was observed at a tonicity equivalent to 0.075 M NaCl. At higher or lower tonicities the oxygen uptake was less than this optimal value. On statistical analysis the differences between the oxygen uptake at a tonicity equivalent to 0.075 M NaCl and those at the following tonicities were found to be significant: 0.047 M ($p = 0.036$), 0.147 M ($p = 0.036$), 0.190 M ($p = 0.03$). Although the differences between the oxygen uptake at 0.075 M and those at the other tonicity values were not significant on statistical analysis, these values nevertheless fall into a smooth curve (Fig. 3). Although a more precise localization of the maximum could be determined if a larger number of studies were carried out, there is little doubt that maximum stimulation occurs at or near a tonicity equivalent to 0.075 M NaCl.

TABLE VI

Effect of varying tonicity on islet tissue slice respiration in phosphate buffer. The media contained varying concentrations of Na_2HPO_4–KH_2PO_4 buffer, pH 7.4; the proper tonicity was attained by adding the appropriate concentration of NaCl

PO_4 concentration (M)	Tonicity of medium (equivalent NaCl concentration) M/l	No. determinations	Average mμl. O_2/μg. protein/hr.	σ
0.066	0.76	8	1.84	0.93
0.066	0.380	8	2.37	0.92
0.066	0.190	8	2.68	1.18
0.066	0.093	8	4.43	0.91
0.033	0.190	8	2.62	0.84
0.033	0.093	8	4.10	1.28
0.033	0.047	8	3.16	1.05
0.017	0.093	8	3.35	0.93

Since 0.066 M phosphate buffer has a tonicity equivalent to 0.093 M NaCl, it is obvious that one cannot simultaneously achieve the conditions for both optimal phosphate and optimal tonicity. In order to achieve the most effective compromise, we have compared the respiration of islet tissue slices at pH 7.4 and at optimal phosphate sub-optimal tonicity (0.066 M Na_2HPO_4–KH_2PO_4 buffer, tonicity equivalent to 0.093 M NaCl) with that obtained at sub-optimal phosphate-optimal tonicity (0.054 M Na_2HPO_4–KH_2PO_4 buffer, tonicity equivalent to 0.075 M NaCl). The oxygen uptake in 0.054 M phosphate buffer was 3.54 mμl. per μg. protein per hour;

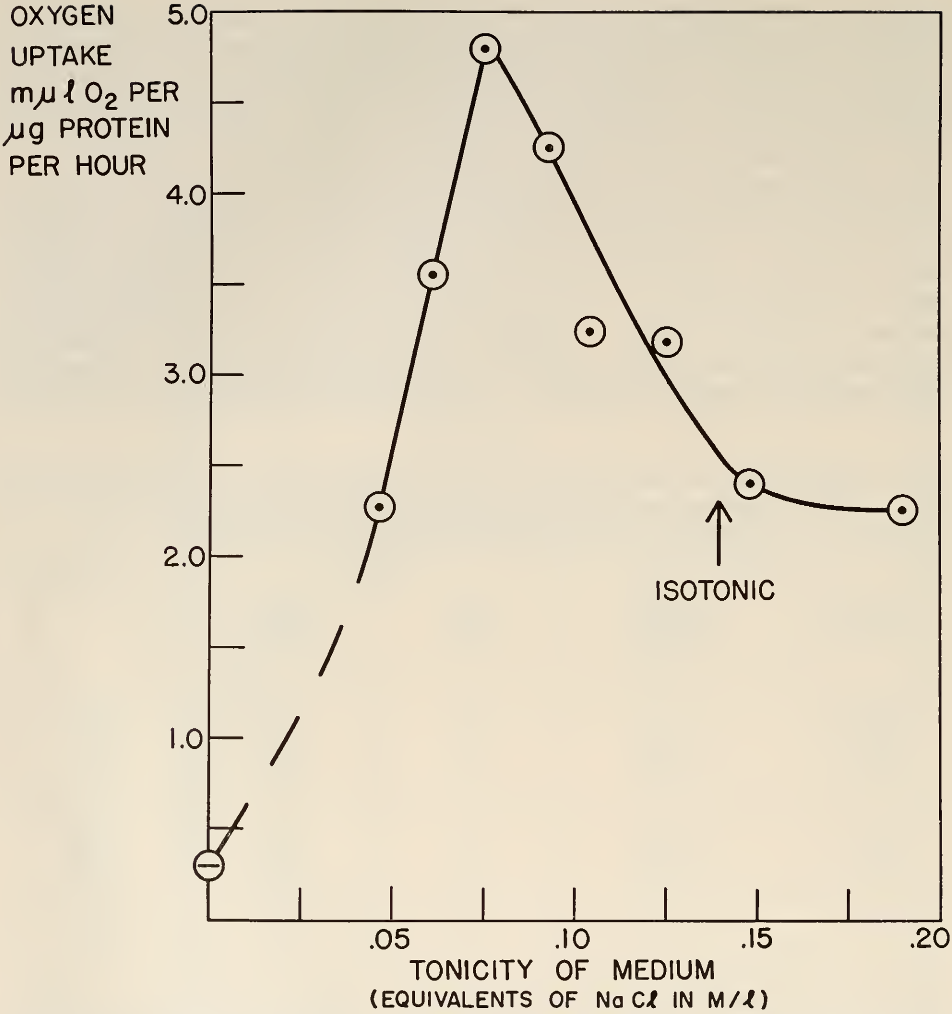

FIGURE 3. Effect of tonicity of the medium on the respiration of islet slices suspended in 0.033 *M* Na_2HPO_4—KH_2PO_4 buffer, pH 7.4. Each of the points represents the average of 8 to 10 individual determinations. The measurements at zero tonicity were carried out in distilled water and therefore this point represents the respiration in the absence of phosphate.

this was 5% greater than the activity in 0.066 *M* phosphate buffer. Although this difference is slight, it does agree with the value that would be expected on the basis of data shown in Figures 2 and 3. A decrease in the phosphate concentration from 0.006 *M* to 0.054 *M* would bring about a 3% decrease in the metabolic activity (Fig. 2), whereas a change in the tonicity of the medium from a sodium chloride equivalent of 0.093 *M* to 0.075 *M* would bring about a 13% increase in the metabolic activity. Therefore in the subsequent studies we used a 0.054 *M* Na_2HPO_4–KH_2PO_4 buffer, pH 7.4, which has a tonicity equivalent to 0.075 *M* NaCl.

The effect of calcium. Media containing a constant amount of phosphate buffer (0.054 M) and varying amounts of calcium chloride ranging from 0.002 M to 0.00005 M were prepared. Since the final tonicity of these solutions ranged from a sodium chloride equivalent of 0.075 M to 0.077 M, the expected changes in metabolic activity as a consequence of tonicity changes would be insignificant. The addition of calcium ion at a concentration of 0.002 M produced a 49% inhibition ($p < 0.001$) of oxygen uptake; at 0.001 M it produced a 25% inhibition ($p = 0.02$) of oxygen uptake; at concentrations of 0.0005 M and 0.00005 M it did not affect the metabolic activity.

The effect of magnesium. The effect of magnesium ion addition was tested both in the presence and absence of added calcium. Magnesium, in concentrations ranging from 0.01 M to 0.002 M, did not affect the metabolism of the islet tissue slices either in the absence or presence of calcium chloride (1×10^{-4} M $CaCl_2$).

The effect of trace metals. A mixture of trace metal salts, constituting the minimal trace element requirements for Neurospora growth (Beadle, 1945), was added to 0.054 M phosphate buffer. The final concentrations of the trace elements per liter of medium were: boron 0.01 mg., molybdenum 0.02 mg., iron 0.2 mg., manganese 0.02 mg., and zinc 2.0 mg. These metals were added as the following salts: $Na_2B_4O_7$, $(NH_4)_2MoO_4$, $FeCl_3$, $MnCl_2$, and $ZnCl_2$. In addition, two other media were prepared in which the concentrations of the trace metals were 10 times and 100 times greater, respectively, than those listed above. The addition of the above trace metals at the minimal concentration did not affect the respiration of the islet tissue slices. When the concentration of each trace metal was increased 10-fold there was a 15% inhibition ($p = 0.44$) of the oxygen uptake; when increased 100-fold, there was a 30% inhibition ($p = 0.05$).

The effect of pyrophosphate. The respiration of islet slices was studied in a medium containing 0.005 M pyrophosphate plus 0.054 M phosphate buffer. The added pyrophosphate can act as a chelating agent and thus remove trace metal ions. Its addition, however, did not affect the respiration of islet tissue slices.

The effect of serum. Samples of toadfish blood were drawn from the gill by venipuncture and the serum separated by centrifugation. The oxygen uptake of islet slices in serum was compared with that in 0.054 M phosphate buffer and found to be 34% lower ($p = 0.007$). On the other hand, when the serum was previously dialyzed against three liters of 0.054 M phosphate buffer for 18 hours at 0° C., the oxygen uptake almost equaled (95%) that in the 0.054 M buffer. Part of this difference may be due to a phosphate ion effect. However, since the tonicity of toadfish serum is considerably greater than the tonicity of 0.054 M phosphate (equivalent to 0.075 M NaCl), the low values obtained in serum, and the higher values obtained in dialyzed serum, can in part be due to tonicity differences. From the results obtained in Figure 3 one would expect that an increase in tonicity to that found in serum would produce a 50% decrease in the oxygen uptake, whereas a 35% decrease was actually found. These results suggest that the addition of serum protein *per se* does not materially affect the respiration of islet slices.

Discussion

It should be noted that maximal stimulation of islet respiration was observed at a tonicity equivalent to 0.075 M NaCl; this is considerably lower than the tonicity of toadfish blood.[3] Similar stimulation of the oxygen uptake of brain homogenates when suspended in hypotonic media has been reported by Elliott and Libet (1942). The activity of the succinic oxidase system is likewise increased in hypotonic media and this stimulation is believed to be a direct effect on the enzyme complex (Tyler, 1954).

The stimulation of toadfish islet metabolism by phosphate is of interest. The addition of phosphate increases the oxygen uptake of brain homogenates (Elliott and Libet, 1942); it also increases the activity of certain isolated enzyme systems (Kearney, Singer and Zastrow, 1955; *cf.* Koeppe, Boyer and Stulberg, 1956). It has been suggested that it may also play a role in the control of respiration (*cf.* Lardy and Wellman, 1952). It would therefore be of interest to determine whether the oxygen uptake of slices of other toadfish tissues is similarly stimulated by phosphate addition, or if this stimulation is limited to islet tissue. Since islet tissue contains large amounts of zinc (Okamoto, 1942), and since high concentrations of the trace metal ions inhibited the oxygen uptake of the islet tissue, the phosphate stimulation could be the result of zinc chelation. It would therefore be of interest to see if pyrophosphate or other chelating agents can substitute for phosphate.

Islet tissue contains about 11% [4] protein and therefore the oxygen uptake of islet tissue slices (3.5 mμl. of oxygen consumed per μg. of protein per hour) would be equivalent to 0.39 cc. per gram of tissue (wet weight) per hour. This observed value is about equal to the reported value for brain brei and greater than that reported for liver slices. Vernberg (1954) found that the oxygen uptake of toadfish brain brei was equal to 0.41 cc./gm./hr.; the Q_{O_2} of toadfish liver slices was equal to 0.27 cc./gm./hr. The Q_{O_2} of the toadfish islet tissue is about 10 times greater than the oxygen utilization by the intact animal. Hall (1929) has reported that the oxygen consumed by the toadfish varies directly with the oxygen tension (between a partial pressure of 0 and 115 mm. of oxygen). At the atmospheric oxygen content he found that the toadfish utilized 0.038 cc. of oxygen per gram of fish per hour. Thus the toadfish islet tissue is very active metabolically compared to the fish as a whole and to the other tissues.

SUMMARY

1. The metabolic activity of toadfish islet slices was measured in a cartesian diver microrespirometer under varying experimental conditions. The effects of pH, specific electrolytes, tonicity, trace metals, and protein addition were studied.

2. The metabolic activity was not affected by varying the pH of the medium between 6.2 and 8.0. The addition of phosphate ion stimulated the respiration. The maximum stimulation was observed when the external medium contained 0.066 *M* phosphate.

3. The respiration of islet slices was increased when the tonicity of the suspending media was reduced; optimal respiration was observed in a hypotonic me-

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[3] Green and Hoffman (1953) have measured the osmotic pressure of blood samples obtained from the heart and found them to be equivalent to 0.14 *M* NaCl; the osmotic pressure of blood samples obtained from the gill were equivalent to 0.19 *M* NaCl. These authors consider the tonicity values of the heart blood samples to be the more accurate, for the gill blood samples may have been contaminated with sea water.

[4] The protein content of 5 samples of islet tissue was measured and found to contain 11.2% protein.

dium with a tonicity equivalent to 0.075 M NaCl; this corresponds to a phosphate buffer concentration of 0.054 M. This is slightly sub-optimal with respect to phosphate ion concentration. Since the metabolic activity of islet tissue slices suspended in 0.054 M phosphate buffer (optimal tonicity but sub-optimal phosphate) is slightly greater than in 0.066 M phosphate buffer (optimal phosphate but sub-optimal tonicity), subsequent studies were carried out using 0.054 M phosphate buffer.

4. High concentrations of calcium (0.001–0.002 M) inhibited the respiration of islet slices. The addition of serum protein, lower concentrations of calcium ion (0.0005 M), magnesium ion (0.0002–0.01 M), and small amounts of trace metals (boron, molybdenum, iron, manganese, zinc) did not stimulate the respiration of islet tissue slices.

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EVIDENCE FOR HORMONE–CONTAINING GRANULES IN SINUS GLANDS OF THE FIDDLER CRAB UCA PUGILATOR

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It is known that in neurosecretory systems the products of secretion are stored in axon terminations where they aggregate in particles or granules (Scharrer and Scharrer, 1954; Welsh, 1955).

Some recent electron microscope studies have shown a constancy in the appearance of such structures in the neurohypophysis of different vertebrates (Duncan, 1955, 1956). The size of these granules varies from 0.1 to 0.3 micron, and in the neurohypophysis of the rat they seem to be bounded by a delicate membrane (Palay, 1955).

Through the differential centrifugation technique for isolation of mitochondria and other particles of the cells, Hillarp, Lagersted and Nilson (1953) and Blaschko and Welch (1953) could obtain a fraction of granules which is responsible for 80 to 90% of the total adrenaline and noradrenaline present in the adrenal medulla of cattle. Further, Hillarp and Nilson (1954) and Blaschko, Hagen and Welch (1955), doing physiological experiments with the separated granular fraction, obtained information concerning the nature of the granules containing the catechol amines. Similar results were obtained for the granules containing vasopressin and oxytocin in the posterior pituitary of the rat (Pardoe and Weatherall, 1955). The observations of the several authors, above cited, strongly support the assumption that the granules have a semipermeable membrane of a lipid or lipo-protein nature. The granules, which are stable in isotonic solutions of saline or sucrose, release their hormone when treated by agents which are known to damage biological membranes.

In the invertebrates, especially among insects and crustaceans, some neurosecretory systems are very well known, and the study of the granules in these systems might give valuable information concerning such storage particles. A good structure for these studies is the "sinus gland" of the crustaceans. A sinus gland in each eyestalk is the storage-release organ for several neurohormones of the crustaceans. They are formed by the axon terminations of neurosecretory cells localized in the X-organ, in the brain and in other parts of the central nerve system (Passano, 1951a, 1951b; Bliss and Welsh, 1952; Bliss, Durand and Welsh, 1954). The axon terminations in sinus glands are filled with granules 0.1 to 0.3 micron in diameter (Potter, 1956) which can be seen in living preparations (Passano, 1952).

The aim of the present work was to show that the granules in sinus glands are really the depots of neurosecretory materials and that they behave like similar structures found in the neurosecretory systems of vertebrates.

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MATERIAL AND METHODS

For this purpose the study of one of the chromatophorotropic hormones stored in sinus glands was chosen. According to the usual technique for isolation and preservation of mitochondria (Hogeboom, Schneider and Palade, 1948) the sinus glands were homogenized in isotonic solutions of sucrose. The homogenates before and after several treatments were injected into test animals for an estimation of the activity of the hormone in the different cases.

Preparation of the homogenates

Sinus glands of the fiddler crab, *Uca pugilator,* from Florida, were used in these experiments. With the aid of a dissecting microscope the sinus glands were isolated from the adjacent tissues immediately after cutting the eyestalk of the crabs, and were placed in solutions of cold 1.3 *M* sucrose, which, according to Abramowitz and Abramowitz (1938), is isosmotic with the blood of *Uca*. In each experiment four or more sinus glands were homogenized in one ml. of isotonic sucrose, in the Elvehjem homogenizer for three minutes. After homogenization more sucrose was added according the requirement of the experiment. A part of the homogenate was then kept at 2° C. until the moment of the experiment, and the remainder was submitted to different treatments. Before being injected into the test animals all homogenates were diluted in isotonic sucrose, or sea water to make the same final concentration. All the procedures were carried out in the cold at 2° C. For details, see below.

Assays

The activity of the black chromatophore-dispersing hormone in the different homogenates was tested in isolated legs of *Uca pugnax*. It was observed that in legs of *Uca pugilator* when they are separated from the body, the black chromatophores disperse gradually. Such dispersion may be explained by a direct effect of light on the chromatophores, since legs isolated and kept in sea water in the dark do not show this phenomenon. A direct effect of light on the chromatophores of *Uca pugilator* has been already observed by Brown and collaborators (Brown and Sandeen, 1946, 1948; Brown, Guyselman and Sandeen, 1949). For this reason in the present experiments the legs of *Uca pugnax* which do not show this behavior were used.

Uca pugnax were destalked 24 hours before the experiment so that at the time of the experiment the black chromatophores were in the stage of maximal concentration. The legs were cut off at the level of the ischial segment, and were placed in 5 ml. of sea water in Syracuse dishes. Each leg received an injection of 0.01 ml. of homogenate, and the dispersion of the black chromatophores was observed every ten minutes for one hour. *Uca pugilator* was used for experiments in which the homogenates were tested in the whole animal. In these cases, each animal received 0.1 ml. of homogenate and the stages of the chromatophores were observed for several hours.

Preliminary attempts were made to remove granules from the homogenates by centrifugation.

All the results are presented in graphs according to Hogben and Slome (1931), where 1 represents maximal concentration of the chromatophores, 5 maximal dispersion, and 2, 3 and 4, intermediate stages.

RESULTS

I. Hormone in granules and in cytoplasm

The homogenate of 4 sinus glands in 1 ml. of 1.3 *M* sucrose was divided; one-half of the suspension received 4.5 ml. of distilled water and was kept at 2° C.; the other half was kept undiluted at the same temperature. After one-half hour, the homogenate in sucrose was diluted with sucrose, and that with added distilled water was diluted with sea water to make a final concentration of 0.02 sinus gland per ml. Every time that distilled water was added to the homogenates, the final dilution was made in sea water; these homogenates throughout the paper will be called "homogenates in distilled water," to shorten the explanation.

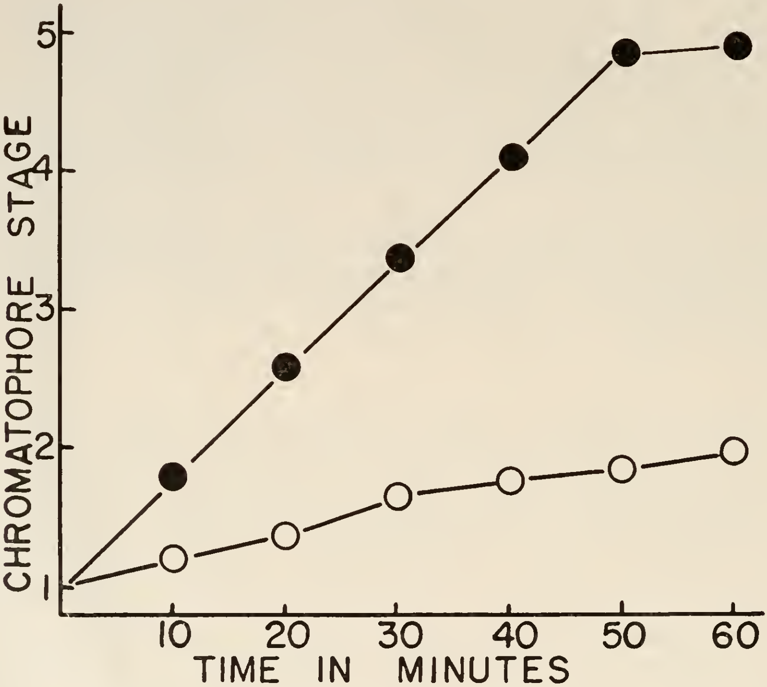

FIGURE 1. Response of the black chromatophores of isolated legs of *Uca pugnax* to injections of homogenates of sinus glands of *Uca pugilator*: ●, homogenates in distilled water; ○, homogenates in 1.3 *M* sucrose. Each point in the graph represents the average of 20 experiments.

As one can see in Figure 1, the homogenate in distilled water caused maximal dispersion of the chromatophores in the legs of *Uca,* and that in sucrose exhibited only a small effect. These results show that in isotonic sucrose the black chromatophore-dispersing hormone is present in large part in a state in which it cannot act; whereas in distilled water it seems to be free in solution and able to induce the dispersion of the chromatophores. This evidence supports the view that the hormone is contained in granules, which in isotonic sucrose remain intact and in distilled water release the hormone into the solution.

If that were the case it would be possible to separate a fraction of granules containing hormone, using the usual technique of differential centrifugation. To avoid the high density of a medium like 1.3 *M* sucrose, 16 sinus glands were homogenized in one ml. of a mixture of 25% of 1.3 *M* sucrose and 75% of sea water, which has been demonstrated to be as effective in preserving the granules as pure sucrose. The homogenate, after dilution to make 10 ml., was centrifuged at 800 × gravity for 30 minutes for separation of unbroken cells, nuclei, etc. No visible sediment was observed after this slow-speed centrifugation, so the whole solution was decanted. Part was set aside, as "solution A," and the rest was centrifuged at 20,000 × gravity for 30 minutes. No sediment was observed this time either. The whole solution was decanted carefully and taken as "solution B." Then one ml. of distilled water was added to the centrifuged tube and was stirred and the tube walls were scraped with a spatula. After 15 minutes 9 ml. of sea water were added to make 10 ml., and this solution was called "solution C." Part of the solution A and B was treated with distilled water and all three solutions were finally diluted to the concentration of 0.02 sinus gland per ml. Both solutions A and B showed the same

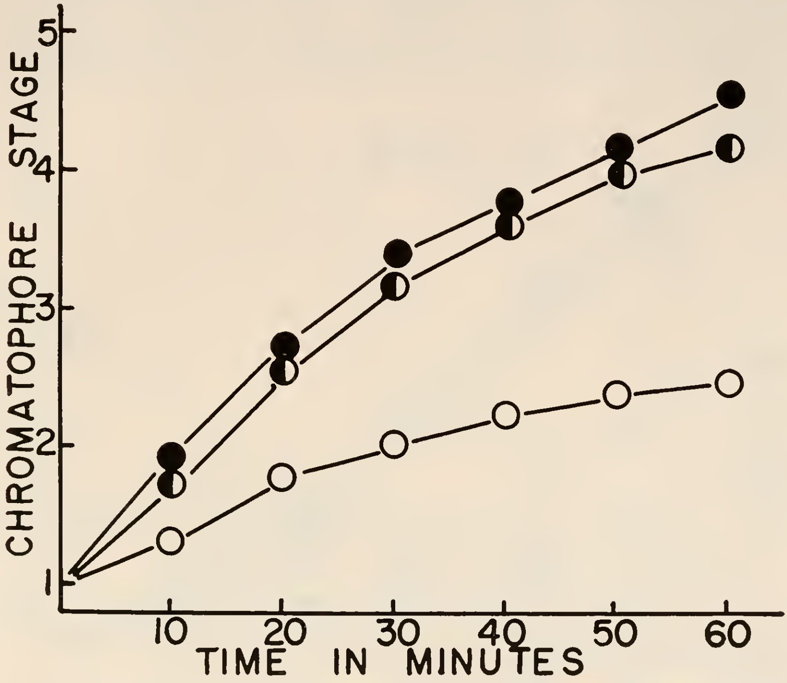

FIGURE 2. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in isotonic sucrose after centrifugation: ●, "supernatant A" (low speed); ◐, "supernatant B" (high speed); ○, "sediment C" (high speed).

effect on the chromatophores of legs of *Uca pugnax*. No significant loss of activity was observed in solution B after the high speed centrifugation. However, solution C, the suspension of a presumably invisible sediment, caused a small effect on the chromatophores (Fig. 2). This fact is indicative of some sedimentation of granules and from these results it is not possible to infer how much of the hormone is present in granules and how much is found free in the homogenate. It is probable that for a complete sedimentation a longer and higher-speed centrifugation is necessary.

An indication of the percentage of hormone contained in granules in isotonic sucrose is given by the analysis of the activity of homogenates in isotonic sucrose and distilled water after a series of dilutions. Figure 3 shows the results of injections of 0.01 ml. of homogenates of 2 sinus glands in 1 ml. of 1.3 *M* sucrose and in 1 ml. of distilled water diluted 10, 100 and 1000 times in 1.3 *M* sucrose and sea water, respectively. It appears that in isotonic solution only less than 10% of the hormone is found free in the suspension, since the effects of the homogenates in sucrose are smaller than those of the homogenates in distilled water, ten times more diluted.

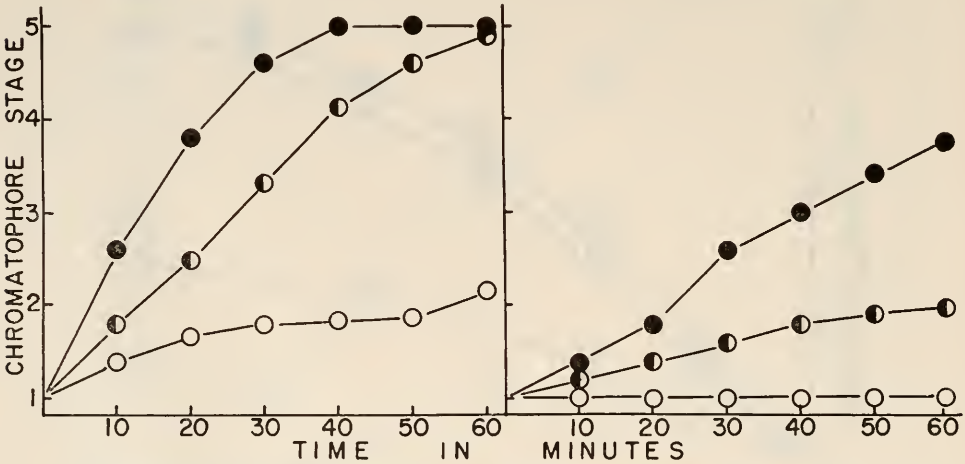

FIGURE 3. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in distilled water (left) and in 1.3 *M* sucrose (right) in different concentrations: ●, 0.2; ◐, 0.02; and ○, 0.002 sinus glands per ml.

II. Effect of several treatments on the release of the hormone

1. Effect of the tonicity of the medium. From the homogenates of 8 sinus glands in 2 ml. of 1.3 *M* sucrose, 7 samples of 0.25 ml. each were separated. The addition of 9.75 ml. of 0.9, 0.8, 0.7, 0.65, 0.32 *M* sucrose was made to a series of 5 tubes and to a sixth, 4.75 ml. of distilled water were added. After one hour at 2° C., the solutions were diluted in 1.3 *M* sucrose to the final concentration of 0.02 sinus gland per ml. and were tested on isolated legs. Figure 4 illustrates the activity of the hormone in the different solutions. As the tonicity of the medium decreases there is a liberation of hormone which, to some extent, is proportional to the concentration of sucrose, from 1.3 to 0.7 *M*. In 0.65 and 0.32 *M* solutions there seems to be a complete release since the activity of the hormone in these two latter concentrations is as great as that of homogenate in distilled water. The action of distilled water after 15 minutes standing is as effective as after 30 minutes. This fact shows that the release in distilled water is rapid. The granules in this respect, like red blood cells and mitochondria under the same conditions, appear to act as osmometers.

2. Effect of different solutions. One group of experiments was performed to determine whether the granules containing the hormone are also stable in isotonic solutions of electrolytes. Samples of 0.5 ml. of homogenates in 1.3 *M* sucrose were held for 30 minutes at 2° C. with the addition of 4.5 ml. of the following: distilled water, sea water, sodium chloride and potassium chloride. The sodium chloride was either isotonic with sea water (0.54 *M*) or isotonic with 1.3 *M* sucrose (0.78 *M*). Figure 5 shows that sea water and isotonic salt solutions produce a large and rapid release of hormone. This fact indicates that the simple dilution in isotonic electrolyte solutions is sufficient to provoke alterations in the granules very similar to those observed by lowering the tonicity of the medium. However, in electrolyte

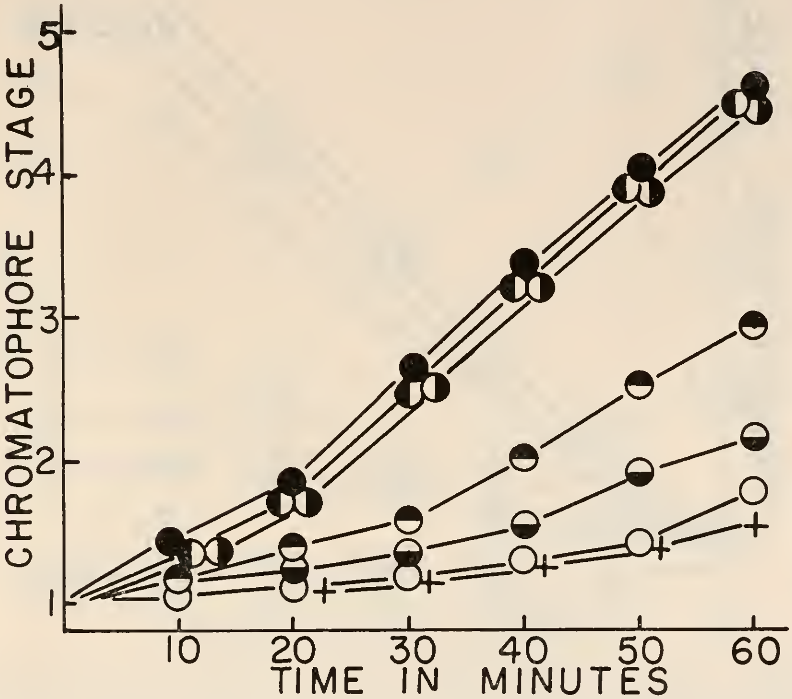

FIGURE 4. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in different concentrations of sucrose solutions: +, 1.3; ○, 0.9; ◒, 0.8; ◓, 0.7; ◑, 0.65; and ◐, 0.32 *M*; ●, homogenate in distilled water.

solutions to which an equal part or a fourth part of isotonic sucrose is added, the granules remain largely intact. The activity of the hormone in these media (Fig. 5) is comparable to that in isotonic sucrose.

In another group of experiments an effort was made to find the best medium for preservation of the granules. Watanabe and Williams (1953) have shown that 2.5% bovine plasma albumin in isotonic potassium phosphate buffer at pH 7 is a good medium to preserve mitochondria of insect muscles. In the following experiments, besides the 1.3 *M* sucrose, a mixture of 25% 1.3 *M* sucrose and 75% sea water was also used, as well as 2.5% bovine plasma albumin in 0.54 *M* potassium phosphate at pH 7. The homogenates of sinus glands in these three media were kept at 2° C. and at different times were diluted in 1.3 *M* sucrose and assayed using legs of *Uca* (Fig. 6). After 6 hours of incubation in these media, the activity of the black chromatophore-dispersing hormone is insignificant, and after 24 hours only a slight effect was observed. That the hormone was preserved in the granules was shown by the following procedure. After 24 hours the homogenates were heated for 5 minutes in boiling water and diluted in sea water. After this treatment all the solutions produced a maximal dispersion of the chromatophores, comparable to that caused by homogenates in distilled water. Thus, the three different media used seem to be equally efficient in keeping the granules intact.

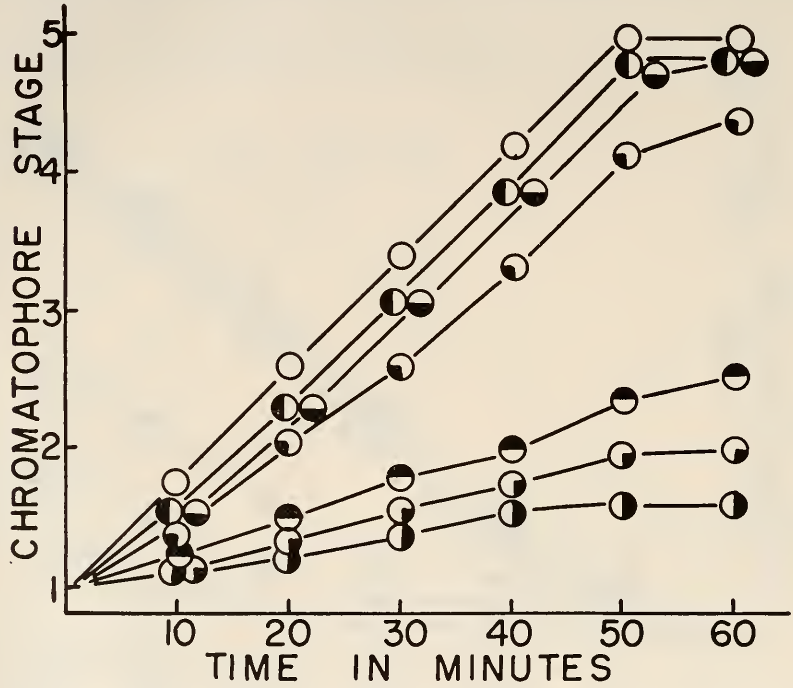

FIGURE 5. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in different solutions: ○, distilled water; ◐, sea water; ◒, 0.78 and 0.54 *M* NaCl; ◔, 0.54 *M* KCl; ◓, ◔, and ◑, NaCl, KCl and sea water in a mixture with 25% of 1.3 *M* sucrose.

3. Effect of heat, and of freezing and thawing. A release of hormone from the granules was observed when homogenates of sinus glands in isotonic sucrose were kept at room temperature for several hours. However, homogenates in 1.3 *M* sucrose when heated for 5 minutes at 70° C. or in boiling water showed only a slightly greater activity than the original homogenate without this treatment (Fig. 7).

Freezing at − 10° C. and thawing to room temperature three times in succession was more effective than heating, but even so, the release of the hormone was not the same as when homogenate was merely diluted in distilled water (Fig. 7).

4. Effect of detergents and digitonin. Detergents and digitonin did not give a complete release of hormone from the granules. Samples of 0.25 ml. of homogenates in 1.3 *M* sucrose were maintained for one hour at 2° C. with 1.75 ml. of 10^{-3} *M* concentration of the following substances: sodium lauryl sulfonate (Duponol); sodium desoxycholate, saponin and digitonin, in 1.3 *M* sucrose. After the required dilution of the homogenates for the bio-assays, the concentration of the detergents and digitonin was 10^{-5} *M*. When control legs of *Uca* or the whole control animal received injections of the detergents and digitonin in such concentration, no effect on the chromatophores was observed. Therefore, the dispersion following the injections of homogenates in sucrose plus detergents is attributed to the hormone present in the solutions.

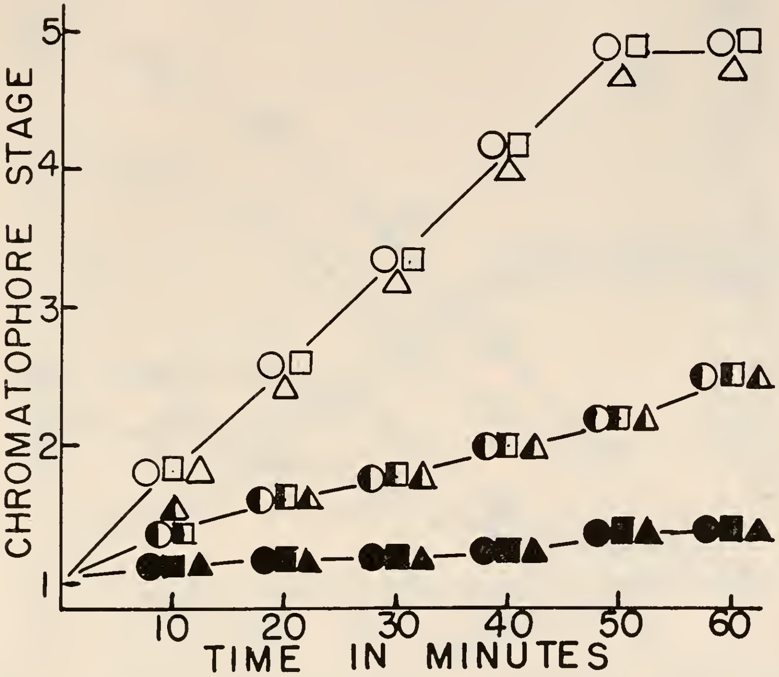

FIGURE 6. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in different media. Circles, homogenate in 5% bovine plasma albumin in 0.54 *M* potassium phosphate buffer; squares, homogenate in 1.3 *M* sucrose; triangles, homogenate in 25% 1.3 *M* sucrose plus 75% sea water. ●, ■, ▲, homogenates kept 6 hours and ◐, ◧, ◭, 24 hours at 2° C.; ○, □, △, after being kept 24 hours at 2° C., the homogenates were heated and diluted in sea water.

The detergents employed and digitonin provoked only a partial release of hormone (Fig. 8). Part of the homogenate plus desoxycholate after one hour of incubation was heated and diluted in sea water, and greater activity was seen after this treatment.

III. Inactivation of the hormone

In some experiments the homogenates of sinus glands in distilled water were injected into the whole crab (*Uca pugilator*). Figure 9 shows the degree and the duration of the dispersion of chromatophores in relation to the concentration of the homogenates. The injection of 0.1 ml. of a homogenate of 0.2 sinus gland per ml., *i.e.*, the injection of an amount corresponding to 0.02 sinus gland, is enough to cause a maximal dispersion of the chromatophores in almost 30 minutes and only four hours later have the chromatophores reached the stage of complete concentration again. It is interesting to notice that the time required for normal dark *Uca* to become pale after eyestalk removal is three to four hours. At all concentrations of homogenates dispersion was found to require less time than concentration of pigment within the chromatophores. The elimination of the hormone seems to be a very slow process. Even an injection corresponding to 0.001 sinus gland (open

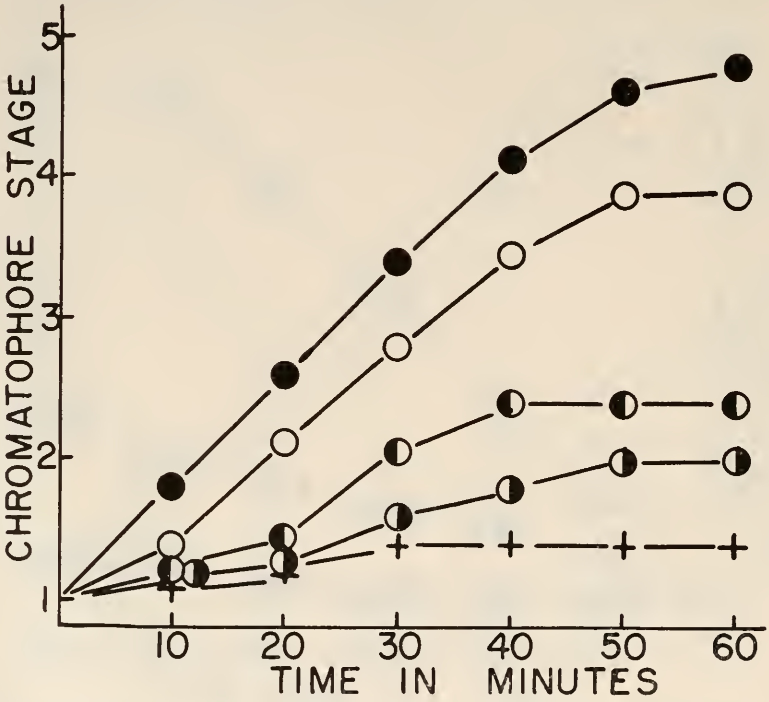

FIGURE 7. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in isotonic sucrose before, +, and after heating at 70° C., ◑, and in boiling water, ◐; and after freezing and thawing, ○. Homogenate in distilled water, ●.

circles in Fig. 9) induces an effect which disappears completely only after three hours.

In order to obtain some information about the inactivation of the hormone, homogenates of sinus gland in distilled water were incubated with extracts of hepatopancreas, hypodermis and muscle and with one ml. of blood of *Uca*. The extracts were prepared by homogenizing one hepatopancreas, the muscle of one claw, and the hypodermis of the branchiostegites separately, in one ml. of sea water. The blood was removed at the junction of the body and the fourth walking leg, with the aid of a glass pipette. The only extract which caused a complete inactivation of the black chromatophore-dispersing hormone was that of hepatopancreas. After one hour of incubation with extracts of hypodermis or muscle, or with blood, at room temperature, no decrease in the activity of the hormone was observed (Fig. 10a).

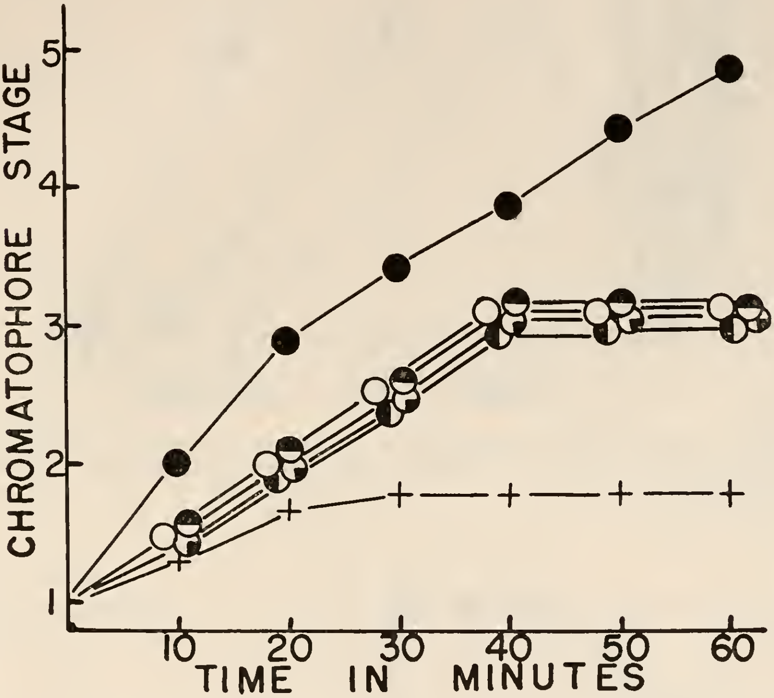

FIGURE 8. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in isotonic sucrose before, +, and after treatment with detergents and digitonin. ◐, Duponol; ◓, saponin; ○, sodium desoxycholate; ◔, digitonin; ●, sodium desoxycholate plus heat and dilution in sea water.

The enzyme in the hepatopancreas responsible for its action might be a proteolytic one, since the same effect was obtained when homogenates of sinus glands in distilled water were incubated at 37° C. for one hour with some crystals of chymotrypsin (Fig. 10b). These results suggest that the black chromatophore-dispersing

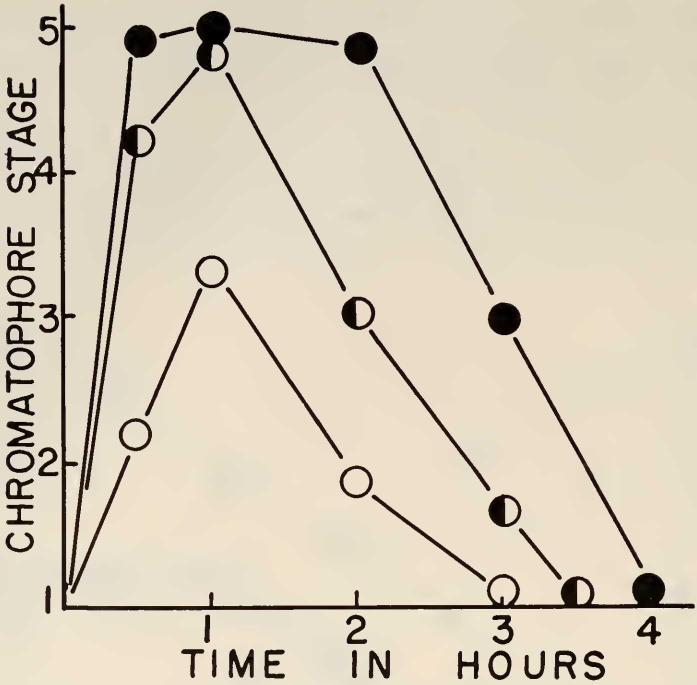

FIGURE 9. Response of black chromatophores of the whole *Uca pugilator* to injections of 0.1 ml. of homogenates of sinus glands in distilled water, in different concentrations: ●, 0.2; ◐, 0.02; ○, 0.001 sinus gland per ml.

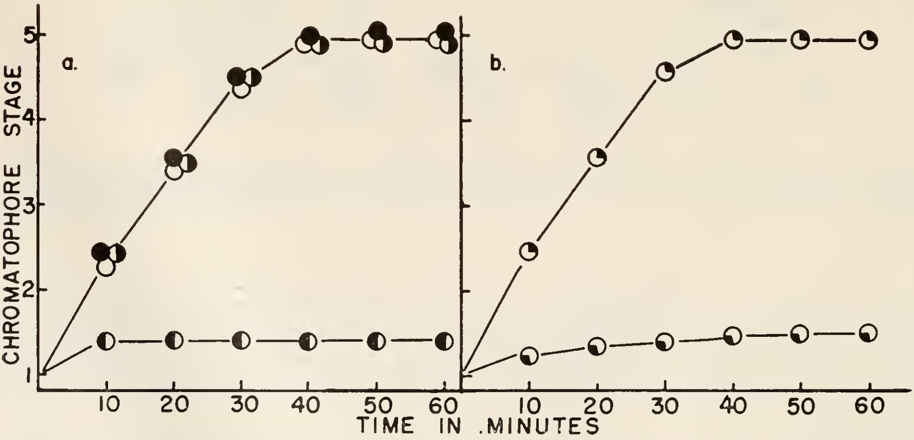

FIGURE 10. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in distilled water before and after incubation with extracts of different tissues for one hour at room temperature and after incubation with chymotrypsin for one hour at 37° C.: ●, muscle; ○, blood; ◑, hypodermis; ◐, hepatopancreas; ◷, distilled water; and ◵, chymotrypsin.

hormone is a polypeptide, but the acceptance of this hypothesis depends upon further experiments.

DISCUSSION

The experiments in section I indicate that in the crab, *Uca pugilator,* the black chromatophore-dispersing hormone is stored in sinus glands within the granules. This assertion is supported by the following observations. First, homogenates of sinus glands in isotonic sucrose have only a small effect on the chromatophores of legs of *Uca pugnax.* These homogenates diluted in distilled water cause a maximal dispersion of the chromatophores, indicating a more or less complete release of the hormone. Second, a sedimentable fraction containing hormone was obtained by centrifugations at the speed of 20,000 × gravity. This centrifugation caused only a partial sedimentation of granules. However the analysis of the activity of homogenates in isotonic sucrose and in distilled water after a series of dilutions shows that the homogenates in sucrose are as effective as those in distilled water ten times more diluted, indicating that only 10% or less of the total amount of hormone is present free in the solution, the other 90% remaining in the granules. Whether this free hormone is already present in sinus glands *in vivo,* or whether it is the effect of the disruption of some granules during the process of homogenizing, is not known. Hillarp, Lagersted and Nilson (1953) have observed that at increased duration of homogenization the catechol content of the granules of the adrenal medulla cells decreases. Berthet and De Duve (1951) have also found that a partial damage to the mitochondria containing acid phosphatase is caused by the process of homogenizing liver tissue. This may be the case with the homogenates of sinus glands.

The effect observed by lowering the tonicity of the medium reinforces the evidence of the presence of the chromatophore-dispersing hormone in granules, and suggests the existence of a semipermeable membrane for the granules. The rapid release of hormone observed when the tonicity of the medium decreases suggests that there is a lysis of the granules, by rapid entrance of water.

The membrane of the granules seems to be freely permeable to ions like sodium and potassium, because the solutions of isotonic sodium chloride, potassium chloride, or sea water cause an immediate and marked release of hormone from the granules. Hillarp and Nilson (1954) have found that the granules of the adrenal medulla can be suspended in sucrose or in certain isotonic electrolyte solutions without a considerable release of catechol amines. Blaschko, Hagen and Welch (1955), however, have observed that in NaCl or KCl an appreciable liberation of adrenaline occurs. Pardoe and Weatherall (1955) also have obtained liberation of vasopressin and oxytocin from granules of the posterior pituitary of rats, by simple dilution in saline of the suspensions of granules in isotonic sucrose. Isotonic saline solutions have been demonstrated to afford only transient osmotic protection for mitochondria of the rat liver (Berthet, Berthet, Appelman and De Duve, 1951; Appelman and De Duve, 1955) and for mitochondria of insect muscle (Watanabe and Williams, 1953). The authors above cited observed also that in media where part of the saline is replaced by isotonic sucrose, the mitochondria are very stable. Similarly, the granules of sinus glands are equally stable in pure sucrose, in a mixture of 25% isotonic sucrose and 75% isotonic salines, and in 2.5% bovine plasma albumin in 0.54 *M* potassium phosphate buffer at pH 7.

Heating, freezing and thawing, and the action of detergents have been proved efficient treatments to release physiologically active substances from granules (Hillarp and Nilson, 1954; Pardoe and Weatherall, 1955). In the case of granules of sinus glands, all these treatments induce a more or less appreciable release of hormone but none of them is sufficient to cause a complete liberation of hormone from the granules.

The hormone in the homogenates in isotonic sucrose after heating, freezing and thawing and after the action of detergents is still present either inside the granules or in such combination that it can not be active. This was proved by the experiments in which parts of the homogenates after these treatments were diluted in sea water, and greater activity was then observed.

These observations may suggest the following hypothesis: that inside the granules the chromatophore-dispersing hormone is found in two forms, bound to a large molecule and as free small molecules. By heating, freezing and thawing and by the action of detergents, the membrane of the granules suffers some disruption, permitting only the passage of the small molecules to the solution. In hypotonic and saline media, which cause a lysis of the granules, all the molecules are present free in the solution. One has to admit also that the hormone is active in both forms, or that once free in the solution, the large molecules disintegrate into the smaller ones. This could explain the different activity of the homogenates of sinus glands in isotonic sucrose after these different treatments.

It is interesting to discuss here the results of Knowles, Carlisle, and Dupont-Raabe (1955) with the chromactivating substances of sinus glands and post-commissure organs of *Leander serratus,* and corpora cardiaca of *Carassius.* By electrophoresis of extracts of these organs they detected the presence of a substance, the "A-substance," which is relatively immobile at pH 7.5 and does not pass through cellophane membranes. This substance concentrates all the red chromatophores of *Leander.* When the extracts are left standing several hours at room temperature, the A-substance disintegrates into others, the a-substances, which have high mobility at pH 7.5 and pass freely through a dialysis membrane. The a-substances affect only the small red chromatophores of *Leander.* They observed also that only the a-substances are released by electrical stimulus of the commissure when the post-commissure organ is in a saline bath.

So, it is reasonable to believe that the dispersing hormone of *Uca* can also be found as large and small molecules and both be active on black chromatophores. But, of course, this is an assumption which depends upon further experiments in this subject.

Heating in boiling water does not cause loss in the activity of the chromatophore-dispersing hormone of *Uca.* Inactivation of the hormone can be achieved, however, by incubation of the homogenates of sinus glands with extracts of hepatopancreas and by the action of the enzyme chymotrypsin. These results suggest that the hormone is a polypeptide.

Carstam (1951) has found that extracts of hepatopancreas of crustaceans and molluscs, and extracts of liver of the guinea pig inactivate the pigment-concentrating hormone of *Leander adspersus,* but he could not obtain the inactivation of the hormone with trypsin. However, Knowles, Carlisle and Dupont-Raabe (1956) have obtained a complete inactivation of the "A-substance" from sinus glands and post-commissure organ of *Leander,* by a crystalline preparation of trypsin and also by a prolonged acid hydrolysis. Östlund and Fange (1956) have suggested that a chromactivating substance from the eyestalk of *Pandalus* could be an aromatic amine, but in personal communication to Knowles and Carlisle (1956) they have stated that their more recent work indicates that this hormone may possibly be a polypeptide. So far, the studies concerning the nature of the chromactivating substances of crustaceans indicate that they are polypeptides. Porath, Roos, Landgrebe and Mitchell (1955) have isolated a melanophore-stimulating peptide from the pig-pituitary gland. Thus, also in vertebrates the chromatophorotropins seem to be peptides.

Carstam (1951) has also obtained the inactivation of the pigment-concentrating hormone by an enzyme present in the hypodermis of *Leander*. In *Uca pugilator*, in hypodermis as well as in the blood, there was not found an inactivating enzyme for the chromatophore-dispersing hormone.

The experiments where the homogenates of sinus glands in distilled water were injected into the whole *Uca pugilator* show that the response of the black chromatophores is a function of the concentration of the hormone. These results give also an idea about the amount of hormone liberated and its way of action in normal crabs. Insignificant amounts of the hormone (corresponding to 0.02 sinus gland) are enough to induce a maximal dispersion of the chromatophores for a long time. This shows that the elimination or destruction of such small quantities of hormone is a slow process. Stephens, Strickholm and Friedl (1956) have also observed that the dispersing hormone in *Uca* was present in the circulating blood of destalked assay animals in discernible amounts for approximately three hours after injection. Hence, it is reasonable to believe that the dispersing hormone is liberated into the blood in small quantities and eliminated by excretory processes without the interference of special enzymes for its inactivation.

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SUMMARY

1. Homogenates of sinus glands in isotonic sucrose cause little dispersion of black chromatophores when injected into legs or whole *Uca*. A liberation of hormone occurs when homogenates of sinus glands in isotonic sucrose are diluted in distilled water. A fraction, sedimentable by high speed centrifugation, when resuspended in distilled water and injected into the test animals, induces a dispersion of the chromatophores. These results support the view that the black chromatophore-dispersing hormone is contained within granules in sinus glands.

2. The release of the hormone from the granules, obtainable by lowering the tonicity of the medium or by dilution in isotonic saline solutions, suggests that the granules possess a semipermeable membrane.

3. The release of the hormone from the granules is increased by heating, by freezing and thawing, and by the action of detergents and digitonin.

4. The black chromatophore-dispersing hormone may be a polypeptide, since it is inactivated by extracts of hepatopancreas and by chymotrypsin.

5. The rate of disappearance of the hormone from the blood of the crab is very slow.

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THE METABOLISM OF STRONTIUM–90 AND CALCIUM–45 BY LEBISTES

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That fishes accumulate mineral elements from the water in which they swim and incorporate these elements into body tissues has recently been demonstrated by the use of calcium-45 and strontium-89 in fresh water and marine fishes of various species (Prosser *et al.,* 1945; Rosenthal, 1956; Lovelace and Podoliak, 1952; Boroughs *et al.,* 1956; Alexander *et al.,* 1956). The rate of incorporation of calcium-45 into the total body and tissues is linear for *Lebistes* and *Salmo* sp. (Rosenthal, 1956; Lovelace and Podoliak, 1952), but bone and osseous tissues incorporate the nuclide at a greater rate than either visceral organs or muscle. In *Lebistes,* the loss of incorporated calcium-45 from the whole body may be described by at least three separate first-order reactions varying from very fast to very slow, probably reflecting the rate of turnover of visceral organs, muscle, and osseous tissues, respectively. In marine fishes, Boroughs *et al.* (1956) have shown that strontium-89, placed in water, is rapidly incorporated into body tissues and the distribution in tissues is similar to that following oral dosage of the nuclide. These investigators also showed that the rate of excretion of a single oral dose of strontium-89 is rapid during the first few days of the experiment. However, the isotope remaining in the body after the first few days persisted at a constant level for a long time.

In view of the reports that small laboratory mammals (Alexander *et al.,* 1956; Comar *et al.,* 1955), man (Turekian and Kulp, 1956) and marine fishes (Boroughs *et al.,* 1957) discriminate against strontium relative to calcium, and since strontium is chemically similar to calcium, it was of interest to determine the uptake and turnover of strontium-90 by *Lebistes* and to compare this information with that previously obtained with calcium-45. The results of this study form the basis for this report.

Materials and Methods

Adult male wild-type guppies, averaging 125 mg. in weight, were obtained from commercial sources. The experimental design and the treatment of animals and tissues for analysis has been described in detail in a previous publication (Rosenthal, 1956).

All samples for radioactivity assay for strontium-90 were counted after a waiting period of 20 days to permit equilibrium between strontium-90 and its yttrium-90 daughter nuclide. The samples were counted with a windowless gas flow counter to less than a 5 per cent statistical error. Corrections for self-absorption of strontium-90 were made when necessary but the 28-year half-life of this nuclide obviated decay corrections. Assay of calcium-45 was performed as previously described, with the same counting assembly.

No attempt was made to differentiate between strontium-90 and its yttrium-90 daughter, and the use of the term strontium-90 throughout this report refers to the combined activities of strontium-90 and yttrium-90 at equilibrium. The efficiency of the counter for strontium-90 and calcium-45 was determined to be 1.5×10^{10} and 1×10^9 counts per minute per millicurie, respectively. The strontium-90 and calcium-45 were obtained from the Oak Ridge National Laboratories in the form of carrier-free salts.

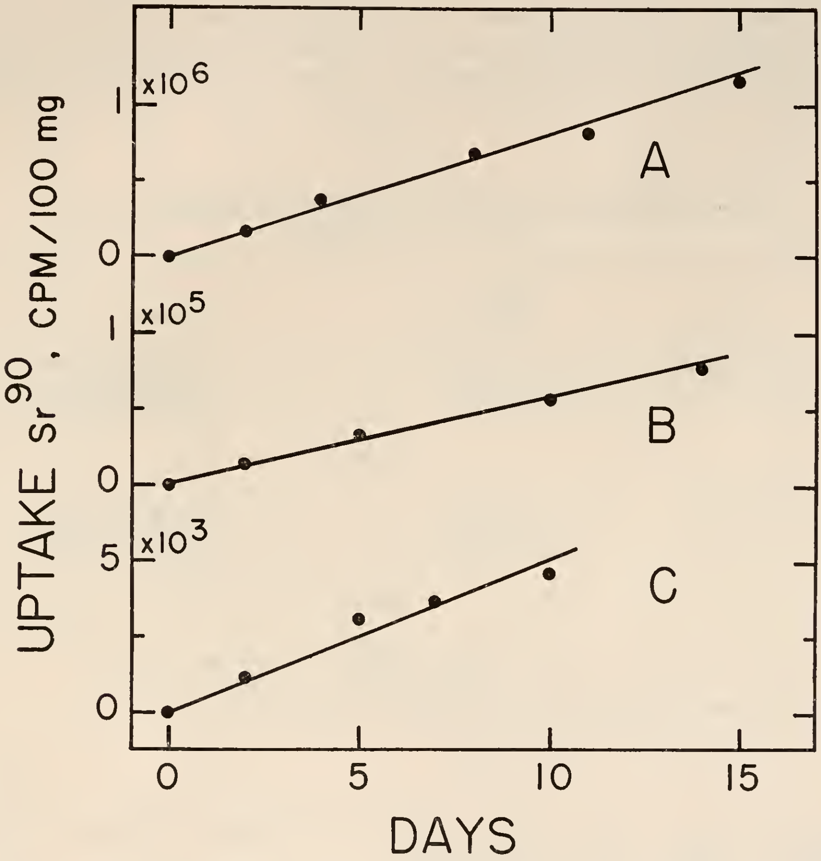

FIGURE 1. Uptake of strontium-90 by male *Lebistes* versus days in water containing the isotope. Each point represents two to four fish. The water activity for Curve A, 1×10^6 cpm/ml., Curve B, 1.7×10^5 cpm/ml., Curve C, 1.1×10^4 cpm/ml.

RESULTS

The rate of uptake of strontium-90 by male *Lebistes* from the water in which they swim was determined by placing the fish in glass aquaria containing 500 milliliters of aged tap water containing the isotope. Distilled water was added daily to compensate for losses of water by evaporation and to maintain the isotope activity of the water within ± 5 per cent during the experimental period.

The results obtained from these experiments demonstrate the rapid incorporation of strontium-90 into the body of the fish (Fig. 1). The incorporation is linear during a 10- or 15-day experimental period for all concentrations of isotope thus far studied, and the uptake of strontium-90 is similar to the data previously obtained with calcium-45 (Rosenthal, 1956). The similarity between the uptake of strontium-90 and calcium-45 is further shown by calculation of a "concentration factor" which relates the logarithm of the rate of incorporation of the isotope in the body of the fish to the logarithm of the activity of isotope in water (Table I). It is apparent that the concentration factors for both calcium-45 and strontium-90 are surprisingly similar within experimental error. These data are markedly different from those obtained for marine fishes by Boroughs *et al.* (1957), who found that *Tilapia* discriminate against strontium-89 relative to calcium. It is conceivable that these differences are due to entirely different mechanisms involving the osmotic physiology of fresh water and marine fishes.

TABLE I

Relationship between rate of uptake and water activity for various nuclides by the body of male Lebistes

Isotope	Water activity cpm/ml.		Concentration factor* ± S.E.
Strontium-90	8.25×10^3		0.70 ± 0.007 (10)**
	1.72×10^5		0.72 ± 0.003 (16)
	1.00×10^6		0.82 ± 0.004 (13)
		Weighted Average	0.75
Calcium-45	8.52×10^3		0.72 ± 0.007 (18)
	9.42×10^4		0.80 ± 0.005 (20)
	7.37×10^5		0.78 ± 0.010 (13)
		Weighted Average	0.77

$$\text{* Concentration Factor} = \log\left(\frac{\text{Uptake in count/min. per 100 mg. per day}}{\text{Water activity in count/min. per ml.}}\right).$$

** The numbers in parentheses indicate number of animals analyzed.

$$\text{S.E.} = \text{Standard error} = \left(\frac{\Sigma d^2}{n(n-1)}\right)^{\frac{1}{2}}.$$

The various organs of the body such as the spine, head, viscera, and muscle also take up strontium-90 in a linear fashion during a 10-day experimental period, but the rate of uptake differs for each organ, as shown in Figure 2. Highly mineralized tissues such as the spine and head accumulate strontium-90 at a greater rate than the total body while soft tissues (muscle and viscera) accumulate less of the isotope. The accumulation of strontium-90 by the tissues of *Lebistes* is qualitatively similar to that of calcium. Although the total body accumulates the same amount of calcium-45 and strontium-90, the ratio of organ isotope concentration to total body isotope concentration for strontium-90 differs significantly from that of calcium-45 in all of the organs studied (Table II). Thus it is apparent that the spine, head

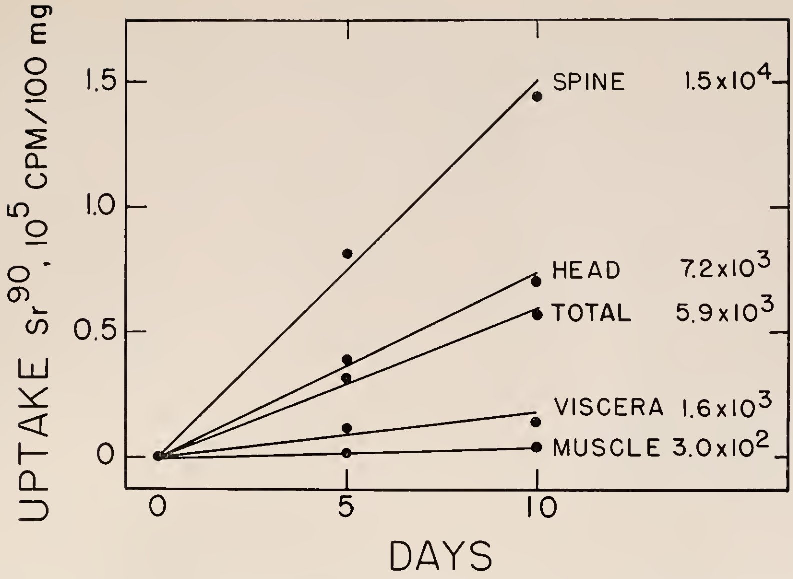

FIGURE 2. Uptake of strontium-90 by various tissues of male *Lebistes* versus days in water containing 9×10^4 counts per minute per milliliter. Values for each tissue represent rate of uptake of strontium-90 in terms of counts per minute per 100 milligrams per day. Each point represents 6 values.

and viscera accumulate significantly more strontium-90 than calcium-45 on a concentration basis. Muscle tissue, on the other hand, tends to incorporate somewhat less strontium-90 than calcium-45. A comparison of the total distribution of strontium-90 and calcium-45 in the various tissues of the body following 10 days of uptake of the isotope from water (Table III) is consistent with the data based on

TABLE II

Relative uptake of strontium-90 and calcium-45 by tissues of male Lebistes

Tissue	Strontium-90*	Calcium-45*	"t"	"P"
Carcass	1.00 ± 0.026** (16)***	1.00 ± 0.045** (20)	—	—
Head	1.28 ± 0.051 (18)	1.07 ± 0.039 (19)	3.29	<0.01
Viscera	0.96 ± 0.118 (18)	0.59 ± 0.087 (21)	2.49	<0.02
Muscle	0.061 ± 0.020 (17)	0.102 ± 0.024 (20)	3.95	<0.01
Spine	2.28 ± 0.10 (15)	1.87 ± 0.10 (16)	3.62	<0.01

* The values represent the ratio $\frac{\text{cpm/100 mg. tissue}}{\text{cpm/100 mg. carcass}}$ derived from 3 to 5 experiments in which the water activity varied from 10^4 to 10^6 cpm/ml. for each isotope.

** Standard error.

*** The numbers in parentheses indicate number of fish analyzed.

$$\text{Students ``t'' value} = \left[\frac{M_1 - M_2}{\left(\frac{\Sigma d_1^2 + \Sigma d_2^2}{N_1 + N_2 - 2} \right)^{\frac{1}{2}}} \right] \left[\left(\frac{N_1 N_2}{N_1 + N_2} \right)^{\frac{1}{2}} \right].$$

TABLE III

Distribution of strontium-90 and calcium-45 in tissues of male Lebistes after 10 days in isotopic water

Tissue	Isotope distribution ± S.E. (per cent of total)		"t"	"P"
	Strontium-90	Calcium-45†		
Carcass	100.0 ± 10.11 (13)*	100.0 ± 2.92 (15)*	—	—
Head	29.9 ± 0.97 (13)	21.3 ± 1.12 (14)	5.71	<0.01
Viscera	13.5 ± 1.87 (13)	7.3 ± 0.48 (14)	3.34	<0.01
Muscle***	2.8 ± 0.29 (12)	3.7 ± 0.31 (14)	2.12	<0.05
Spine	6.9 ± 0.61 (12)	6.2 ± 0.36 (14)	1.02	—
Remainder**	46.9 ± 2.24 (12)	61.5 ± 2.32 (13)	4.36	<0.01

* The numbers in parentheses indicate number of animals analyzed.
** Calculated by difference.
*** Muscle tissues estimated to comprise 40 per cent of body weight.
† From Rosenthal (1956).

concentration shown in Table II. The apparent discrepancy for the similarity between the distribution of calcium-45 and strontium-90 in the spine (Table III) and the relative uptake of the two isotopes by the spine on a concentration basis is due, in all probability, to our inability to always remove the entire spine from these small fishes. This unavoidable error introduces some uncertainty into the distribution data for the spines.

The head and "remainder" (skin, scales and fins) account for 77 per cent of the total body strontium-90, while these tissues account for almost 83 per cent of the total body calcium-45. Muscle contains the smallest proportion of the total body strontium and calcium (2.8 and 3.7 per cent, respectively) while occupying about 40 per cent of the total body weight. The spine, representing less than 3 per cent of the body weight, contains between 6 and 7 per cent of the total calcium and strontium nuclides, respectively. This comparison between the distribution of strontium-90 and calcium-45, under the same experimental conditions, indicates that the head and viscera incorporate, respectively, 25 per cent and 46 per cent more strontium-90 than calcium-45, while muscle and the remaining tissues accu-

TABLE IV

Relative proportions of tissues of male Lebistes

Tissue	No. of determinations	Per cent of body weight
Head	39	20.6 ± 0.22*
Viscera	38	12.3 ± 0.37
Spine	21	2.8 ± 0.07
Remainder**	39	24.4 ± 0.25
Muscle	—	40.0 (estimated)

* Standard error.
** Remainder includes skin, fins, scales and is calculated by difference assuming that muscle comprises 40 per cent of body weight.

mulate about 25 per cent less strontium-90 than calcium-45. These differences in tissue uptake of the two nuclides are statistically significant and further accentuate subtle differences in the metabolism of these two elements.

Since the relationship of the weight of organs to body weight has not, to my knowledge, been previously determined or published, for *Lebistes,* the relative proportions of the various tissues analyzed in this study are shown in Table IV.

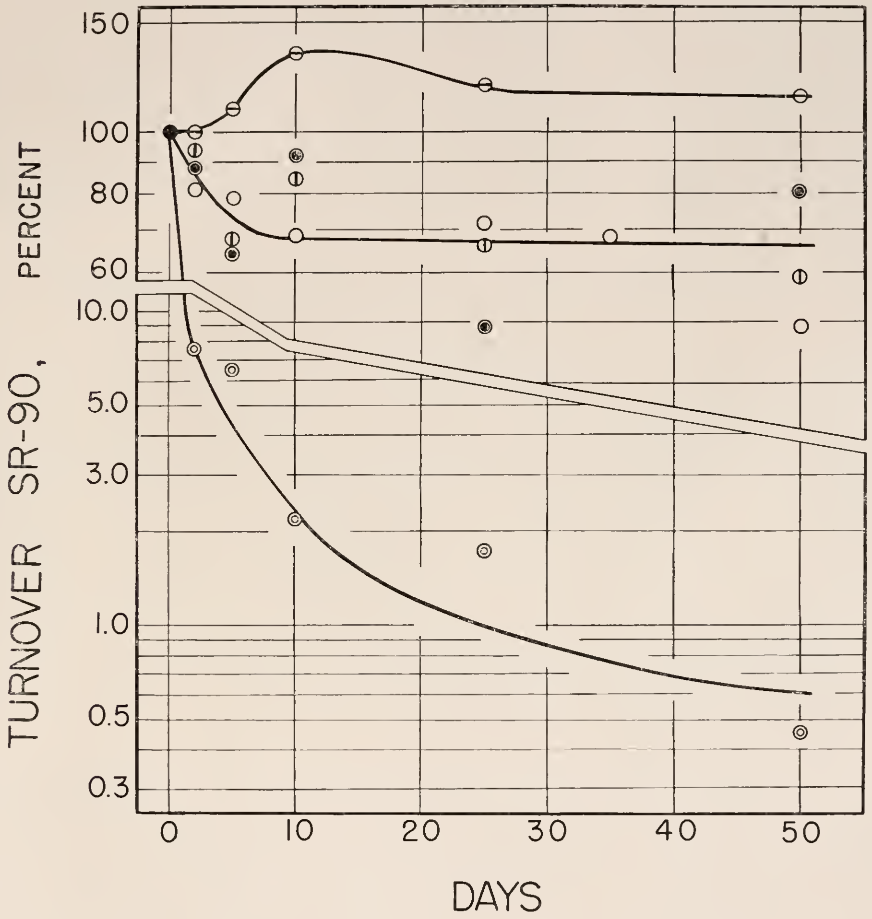

FIGURE 3. Turnover of strontium-90 by various tissues of male *Lebistes* versus days in water containing no isotope. Each point represents 4 to 11 values obtained from 3 experiments. ⊖, spine: ○, body: ●, muscle: ⦶, head: ◎, viscera. The fish contained about 10^4 cpm/100 mg. on day zero of turnover.

The rate of turnover of strontium-90 by the body and tissues of *Lebistes* was determined by first placing the animals in isotope-containing water for 10 days in order to incorporate sufficient radioactivity into the tissues. After this period the fishes were transferred to isotope-free water which was changed periodically and they were sacrificed at suitable intervals previously described in detail (Rosenthal, 1956). During a 50-day experimental period, the loss of strontium-90 from the total fish could be resolved into two components that may be described by first order reactions (Fig. 3). The first component, turning over rapidly with a biological half-life of about 8 days, represents loosely-bound strontium-90 in visceral tissues of the body. This is somewhat longer than the three-day half-life for the rate of turnover of the fast component with calcium-45 (Rosenthal, 1956). The loss of strontium-90 by the viscera is extremely rapid, so that 92 per cent of the radioactivity is lost during the first two days of the experiment. The second component has an exceedingly long half-life of about two years or more. During a similar experimental period with calcium-45 (Rosenthal, 1956) three components with biological half-lives of 3 days, 137 days, and 309 days were apparent which reflect the turnover rates of visceral tissues, muscle and carcass, respectively. The absence of an intermediate component for strontium-90 is primarily due to the very slow turnover rate of strontium-90 by muscle tissue, and to a lesser extent, the head.

TABLE V

Distribution of strontium-90 and calcium-45 in tissues of male Lebistes after 40–50 days of turnover in non-isotopic water

Tissue	Isotope distribution ± S.E. (per cent of total)		"t"	"P"
	Strontium-90 (50 days)	Calcium-45 (40 days)†		
Carcass	100.0 ± 11.2 (5)*	100.0 ± 5.48 (8)*	—	—
Head	42.7 ± 5.16 (5)	34.4 ± 2.08 (7)	1.69	>0.15
Viscera	0.2 ± 0.06 (5)	0.5 ± 0.04 (5)	2.12	>0.05
Muscle***	3.0 ± 0.87 (5)	2.6 ± 0.46 (5)	0.56	NS
Spine	21.0 ± 4.47 (5)	19.4 ± 1.04 (7)	0.41	NS
Remainder**	33.1 ± 7.79 (5)	43.1 ± 2.38 (6)	1.33	>0.20

* The numbers in parentheses indicate number of animals analyzed.

** Calculated by difference.

*** Muscle tissue estimated to comprise 40 per cent of body weight.

† From Rosenthal (1956).

It is of interest to note that muscle tissue strontium-90 with a biological half-life of about two years as calculated from the last 25 days of the experiment, is lost in a manner similar to that of the total body. This is in contrast to the biological half-life for muscle of 137 days as determined previously for calcium-45, and it would appear that the metabolism of the two elements differs in muscle tissue. The exceptionally slow turnover of strontium-90 in muscle tissue of marine fishes has recently been observed by Boroughs *et al.* (1956).

The spine, which consists not only of mineral matter but also of intervertebral cartilage, tendon, and organic bone matrix, continues to incorporate the isotope for about 10 days after the fish is placed in isotope-free water. The additional nuclide must be derived from a redistribution of isotope from soft tissues such as viscera. Similar data were obtained with calcium-45 (Rosenthal, 1956). The accumulated isotope does not remain fixed in the spine, however, but is subsequently lost and a new equilibrium consistent with that of the mineral component of bone becomes established. The biological half-life of strontium-90 in the spine, calculated during the last 25 days of the experiment, may be estimated to exceed two years, a value consistent with the biological half-life of calcium-45 previously determined (Rosenthal, 1956). The additional increase of the strontium-90 and calcium-45 in the bone and its relatively rapid loss may represent a rather labile binding-site (T½ = about 50 days) for bone formation.

The distribution of strontium-90 in various organs and tissues of the body after 50 days in isotope-free water is compared with the distribution of calcium-45 after 40 days in isotope-free water (Table V). It is interesting to note that the distributions of both isotopes at the end of 40 days of turnover for calcium-45 and 50 days for strontium-90 are not significantly different. A comparison of the rate of turnover of strontium-90 by the tissues, shown in Figure 3 of this report, with the rate of turnover of calcium-45 previously published (Rosenthal, 1956) indicates that the similarity of distribution of both isotopes at these particular time intervals is coincidental. Extrapolation of the turnover rates for both isotopes indicates that the head, muscle and spine would retain a greater proportion of the body strontium-90, while the viscera and "remainder" would contain less strontium-90 throughout the life of the fish.

Discussion

It is apparent from these studies that fresh water fishes accumulate strontium-90 from the water in which they swim and that the rate of uptake is similar to that of calcium-45. Moreover, we have recently shown that the rate of uptake of strontium-90 and calcium-45 by other fresh water fishes (*Danio* and *Tanichthys*) is similar to the data we have obtained with *Lebistes*. These data differ from the studies of Boroughs *et al.* (1957), who found that marine fishes discriminate against strontium-89. This apparent disagreement may be due to marked differences in osmotic regulation between marine and fresh water fishes. On the other hand, discrimination of strontium isotopes relative to calcium by small laboratory mammals appears to be well documented (Comar *et al.*, 1955; Turekian and Kulp, 1956; Comar *et al.*, 1956). Comar *et al.*, (1956) have indicated that the processes of major discrimination, in rats, are decreased absorption of strontium from the intestinal tract and increased urinary strontium excretion, processes which cannot be measured directly in small fishes. These two processes would tend to limit the quantity of strontium entering the body and its retention in the body, but in fishes, the gills play a major role for the absorption and excretion of mineral elements. The similarity of the "concentration factor" (Table I) for both nuclides by the total body of the fish indicates no discrimination of strontium-90 by these fishes. It is possible that subtle differences between strontium-90 and calcium-45 uptake by fishes may become apparent by the use of differential methods in which both nuclides are present in the same medium. These and other aspects of the problem are under investigation.

The rate of excretion of strontium-90 by the total body and tissues (except viscera) of *Lebistes* is slower than that of calcium-45. The data appear to be in contrast with the report by Lengeman (1957), who showed that rat bones, *in vitro*, lose more strontium-90 than calcium-45. Since the accumulation and retention of mineral elements into bone depend on the rate of bone formation and sequestration of elements into slowly exchanging bone matrix, comparisons between such diverse biological systems may be hazardous. Nonetheless, tissues of *Lebistes* rich in calcium, such as the spine and head, accumulate and retain a larger percentage of the total body strontium-90 than that of calcium-45, in accord with the studies of Lengeman (1957) and Comar *et al.* (1956) for rats. Although no explanation is offered at this time concerning the mechanism of incorporation of alkaline earth elements from water by fishes, the similarity of uptake of calcium-45 and strontium-90 by fresh water fishes (Rosenthal, 1957) indicates a fundamental and essentially similar process.

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SUMMARY

1. The uptake of strontium-90 by male *Lebistes* from the water in which they swim is linear with time for the total carcass and tissues studied. Tissues containing high concentrations of calcium accumulate more strontium-90 than soft tissues. The rate of turnover of the nuclide varies from very fast to very slow according to the type of tissue. The whole body, head and spine retain strontium-90 for long periods of time (T½ = 600 days) while viscera loses the isotope rapidly (T½ = 8 days).

2. A comparison between strontium-90 and calcium-45 uptake and turnover by male *Lebistes* are qualitatively similar but significant quantitative differences are apparent.

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THE MOLTING CYCLE OF THE SPINY LOBSTER, PANULIRUS ARGUS LATREILLE. IV. POST-ECDYSIAL HISTOLOGICAL AND HISTOCHEMICAL CHANGES IN THE HEPATO-PANCREAS AND INTEGUMENTAL TISSUES[1]

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Following molt, the major tasks which confront the crustacean are growth of the soft tissues and the continued accretionary growth and hardening of the skeleton by deposition of mineral salts therein. In spiny lobsters of 80–89 mm. carapace length, weight stability is not achieved until 28–35 days following molt (late Stage C) (Travis, 1954). This is a period at which the skeleton is fully hardened, water content is normal, and presumably growth of the tissues is fairly stable. During the early postmolt period, however, when rapid accretionary growth and calcification of the skeleton are occurring, marked changes are observed in the hepatopancreas and integumental tissues. Accordingly, the present paper will be concerned with those marked changes in the hepatopancreas and integumental tissues which occur concomitantly with the development and calcification of the post-exuvial layers of the skeleton.

Materials and Methods

Animals

Male and female spiny lobsters ranging in carapace length from 80–89 mm. were obtained and handled as previously described (Travis, 1954).

Designation of stages in the molting cycle

Stages of the molting cycle were designated by time intervals, in days, as previously described (Travis, 1955a) and by the method of Drach (1939). For *Panulirus argus,* Stage A through C encompasses a period of approximately 51 days during the summer months. Stage A, immediately following molt and the stage in which the principal layer begins to be deposited, has a duration of approximately 24 hours or one day. Stage B, beginning calcification, continued thickening of the principal layer and preliminary hardening of the skeleton, is approximately six days

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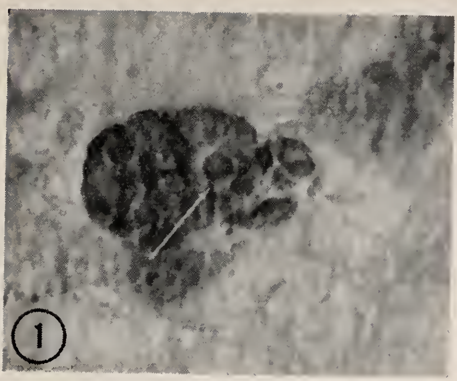
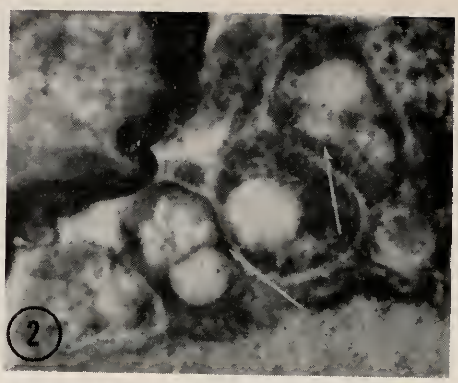
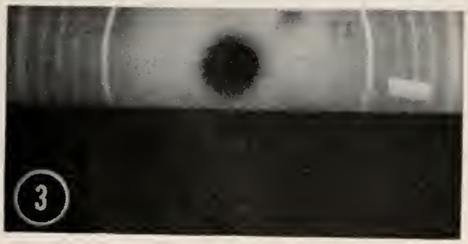
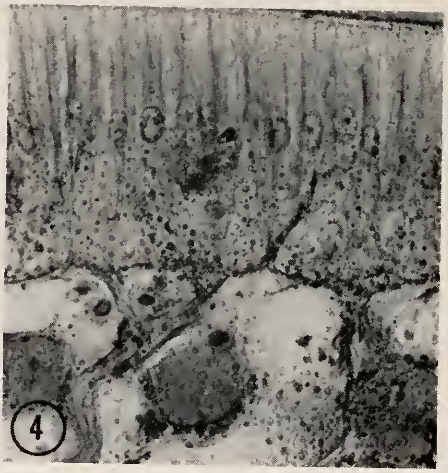
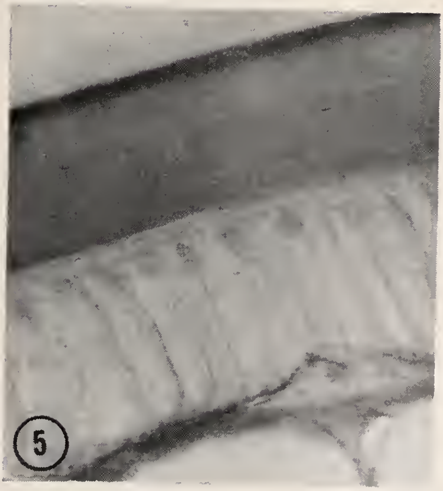
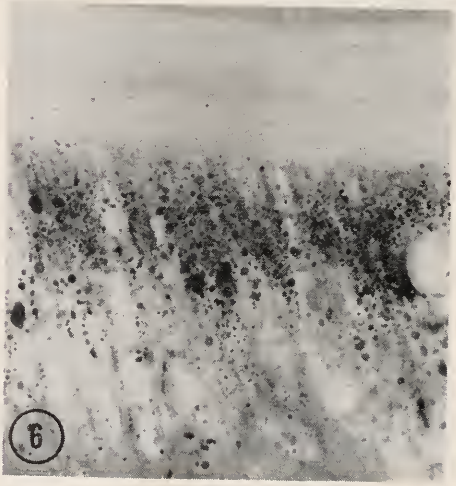












Figs. 1–6.

in length, existing from two through seven days following molt. Stage C, a stage in which the principal layer and the new membranous layer are completed and in which the skeleton is completely hardened, is the longest period of the molting cycle (44 days), existing from approximately the eighth day through the fifty-first day following molt.

Histological and histochemical methods

For the histological and histochemical studies, pieces of integument and integumental tissues were removed from the carapace of *Panulirus* (see Figure 1; Travis, 1955a) on each of eight consecutive days following molt. Likewise the right posterior lobe of the hepatopancreas was removed on each of seven consecutive days following molt. Tissues from three animals were used to represent each of these days with the exception of the first, fourth, and eighth day following molt for the integumental tissues. In these cases, tissues from one animal were used.

Most integumental tissues were embedded in celloidin and cut at 10 μ. The posterior lobe of the hepatopancreas was embedded in paraffin and cut at 8 μ, with the exception of hepatopancreatic tissues fixed and embedded for lipid detection.

Portions of the integument and hepatopancreas fixed in Helly's and alcoholic Bouin's fluid were stained by the following methods:

1. Mallory's triple stain
2. Periodic acid Schiff (PAS) of McManus, as described by Lillie (1948)
3. Bensley and Bensley's method (1938), for demonstrating muco- or glycoprotein by means of toluidine blue (see Travis, 1955a).

For detection of calcium deposits, portions of the skeleton were fixed in nine parts of 95% alcohol and one part of 40% formaldehyde, and were stained with the following:

1. Mallory's triple stain
2. Schmorl's purpurin (Lillie, 1948)

FIGURE 1. A reserve cell, "mulberry"-like in appearance, of the sub-epidermal connective tissue from an animal three days following molt. Note lumpy or stainable balls of material (arrow) of mucopolysaccharide as well as calcium. 800 ×.

FIGURE 2. Large oval reserve cells of the hepatopancreas showing the presence of large vacuoles (arrows), some of which contain flaky or granular-like stainable material while others appear clear, a condition observed from five through seven days following molt and possibly correlated with a marked decrease in mucopolysaccharide and calcium. 800 ×.

FIGURE 3. X-ray diffraction photograph taken of dry powder obtained from triturated pieces of the area of softening. The presence of calcite lines reveals that calcium carbonate is present in the spiny lobster skeleton as calcite, not aragonite or amorphous calcium carbonate.

FIGURE 4. Glycogen distribution in the epidermis of the outer integument and sub-epidermal connective tissue at one day postmolt. Note small number of glycogen granules localized in the proximal half of the outer epidermal cells. At this same time abundant amounts are concentrated at the bases of the epidermal cells of the inner integument. 760 ×.

FIGURE 5. Note that at two days postmolt glycogen has completely disappeared from the epidermis of the inner integument and is not observed again in this tissue during the entire postmolt period. 760 ×.

FIGURE 6. The heavy concentration of glycogen observed in the distal half of the outer epidermal cells during a period of two through four days following molt. Little glycogen at this time is present in the sub-epidermal connective tissue. 760 ×.

3. Alizarin red S (Manigault, 1939)
4. Von Kossa's method (Lillie, 1948). Before following this procedure, tissues were washed in 5% aqueous KNO_3 for five minutes or more to remove the chloride present. With Von Kossa's method, the silver from the silver nitrate is precipitated as phosphate on the surface of calcium phosphate granules. The silver phosphate is reduced in the presence of light to metallic silver, forming a black crust on the surface of calcium phosphate granules. Mallory (1942) states that calcium carbonate granules become coated with silver carbonate, which in sunlight gives off CO_2 and leaves a black silver oxide on the surface of these granules. These reactions may not occur in the presence of organic substances (Lison, 1953) nor in the presence of quantities of chloride (Lee, 1946).

   Parts of the epicuticle, pigmented layer and principal layer appear either black or brown with Von Kossa's method. Cameron (1930) pointed out that the most recent calcium deposits in teeth appear dark brown by this method, while the older calcified layers appear lighter brown. Some calcified areas, he noted, did not stain at all. It should be pointed out that this method has the great advantage over alizarin and purpurin in that it enables one to visualize calcium deposits in granular form. It is, therefore, excellent for the detection of skeletal deposits whereas the latter methods are not.
5. Microincineration (Scott, 1933) was used to confirm the presence of calcium deposits detected by the stains mentioned above. The white calcium ash under dark-field illimination appeared in the same areas as indicated by the stains. To confirm this as being calcium ash, the gypsum test was used.

   For detection of calcium in the hepatopancreas, tissues were fixed in the same fashion as the integumental tissues and were stained with alizarin red S.

For detection of alkaline phosphatase, integumental and hepatopancreatic tissues were fixed in cold 80% alcohol, and embedded in paraffin. Alkaline phosphatase was determined by the method of Gomori (1941). Control sections were made using the incubating medium without added substrate. This method is extremely useful for the detection of calcium deposits. In control sections which do not show the presence of alkaline phosphatase, calcium deposits, if present, show up remarkably well.

Only the hepatopancreas was used for the detection of lipids. Portions of the tissue, in this case, were fixed in 10% neutral formalin and were imbedded in carbowax (method of Blank and McCarthy, 1950), cut at 10 and 15 μ and stained for lipids with Sudan black B.

Observations

The Postmolt Animal (Stage A and B)

1. *The integument and integumental tissues*

   a. *Tissues*

During the early post-ecdysial period (Stage A and B) pieces of exoskeleton with attached integumental tissues were removed from the lateral portion of the carapace (see Fig. 1; Travis, 1955a). The lateral portions of the carapace of

Crustacea, as one will recall, are folded in such a way that there is an outer epidermis and integument, (the outer integument being in contact with the surrounding sea water) as well as an inner epidermis and integument (the inner integument facing the gills, in contact with the sea water in the branchial chamber). Sections of the exoskeleton with attached integumental tissues indicate that the epidermal cells of the outer integument remain extremely long and attenuated and indeed fibrillar in nature. This condition is similar to that observed in the late premolt animal. The markedly fibrillar nature of the outer epidermis, however, is apparent in all stages of the molting cycle. Nuclei in these epidermal cells of the outer integument are central (Fig. 4) whereas those of the inner integument are more distal in location (Fig. 5). The inner epidermal cells, also somewhat fibrillar in nature, remain about half the length of the outer epidermal cells during all stages of the molting cycle (see figures from Travis, 1955a).

Both the outer and inner epidermis, during the early postmolt period (Stage A and B), show a gamma metachromasia (pink-purple) with toluidine blue, indicating the presence of a glyco- or mucoprotein. The presence of phosphatase, glycogen, and calcium in these tissues during the early post-molt period will be discussed in a subsequent section of this paper.

As was pointed out (Travis, 1955a), the sub-epidermal connective tissue is of a loose spongy type.

The large oval reserve cells, described as "protein cells" by Cuénot (1893) and resembling Leydig Cells, Type I (Kükenthal, 1926–1927), constitute by far the most prominent and most interesting cell types within this sub-epidermal connective tissue. These reserve cells vary greatly in structural appearance during the molting cycle. When storing reserves they become greatly swollen and may take on a "mulberry" appearance (Fig. 1). When devoid of reserves they may decrease in size with their vacuoles becoming clear or containing flaky or granular-like stainable material. Since the reserve cells are found within the tissue spaces among other Leydig cells, they should not, perhaps, be considered as permanent structures within this tissue. This has become somewhat clearer from the work of Sewell (1955), in which he points out that the origin of these reserve cells, which he calls "lipo-protein cells," in *Carcinus* is from amoebocytes and that possibly they revert to amoebocytes following molt. This suggestion could account for their cyclic peaks and declines in size and abundance, and changes in structural appearance at daily intervals during the early postmolt period of *Panulirus,* as indicated below.

For the sake of comparison, the reserve cells in *intermolt animals* (*late Stage C*) constitute the most prominent cell-types within the connective tissue. They are large oval cells, vesicular in nature, with a capsule-like envelope of cytoplasm and a peripheral nucleus (see figures; Travis, 1955a). They range in size from 24–51 μ with an average size of 32 μ. After alcoholic Bouin's fixation they stain blue-gray with Mallory's triple stain, the vacuoles in this case containing blue-gray flaky or granular-like material; with the PAS method the entire cell is a deep pink-purple color; with toluidine blue these cells stain either blue-gray or green-gray. After A–F (alcoholic formaldehyde) fixation they similarly stain blue-gray with Mallory's triple stain, but do not stain with alizarin red S, purpurin or the Von Kossa method. With these three latter stains the reserve cells could be easily overlooked.

Similarly, for the sake of comparison reserve cells in *premolt animals* (*Stage D*) range in size from 30–51 μ with an average size of around 40 μ. Structurally, they maintain their oval appearance but stainable material within the cells is lumpy and might well be described as consisting of rather discrete spheres (Fig. 1). After alcoholic Bouin's fixation, the cells again stain blue-gray with Mallory's but do not show clearly the speres of stainable material; with PAS they again stain deep pink-purple and show clearly the discrete balls of material; with toluidine blue the balls of material are yellow-green in color and refractile in nature. Following A–F fixation, the cells again fail to stain with alizarin red S, purpurin and the Von Kossa method.

From one through seven days following molt (Stage A and B), the reserve cells appear to undergo cyclic peaks and declines in size and abundance, and the storing of reserves.

At *one day* following molt (Stage A) the reserve cells remain approximately the same size as those observed in the premolt animal, a range in size from 32–48 μ and an average size of 36 μ. However, there would appear to be a slight decrease in number. Following alcoholic Bouin's fixation, they stain in much the same fashion as that observed in the premolt animal, although the spheres of stainable material are not as apparent. They fail to stain, following A–F fixation, with the same stains mentioned in the premolt animal.

On the *second day* following molt (beginning of Stage B) the reserve cells have greatly decreased in number and size. They range in size from 12–29 μ with an average of around 19 μ. When observed, their vacuoles are clear and vesicular, lacking the lumpy balls of material, with the exception of very small spheres at their periphery. They stain in a similar manner to those observed on the first day with the exception of the fact that a few show a very small number of calcium granules after the Von Kossa method.

On the *third day* the reserve cells again are present in great numbers, comparable to the condition observed in the premolt animals. They range in size from 32–45 μ with an average size of around 38 μ, which compares favorably with the average size observed in the pre- or intermolt animal. The reserve cells at this time take on an irregular "mulberry" appearance by enclosing large stainable spheres of material within their vacuoles (Fig. 1). Further, their staining properties change markedly. Following Bouin's fixation, they again stain blue-gray with Mallory's, deep pink-purple with PAS and yellow-green with toluidine blue. Following A–F fixation, they stain for the first time, a brilliant orange-red with Mallory's scarlet with alizarin red S and purpurin, and yellow-brown with Von Kossa's method. This indicates that not only is muco-polysaccharide, possibly muco- or glycoprotein, bound by these cells at this time but that they are filled with calcium, which is distinctly apparent with stains used for this purpose.

Interestingly enough, by *the fourth day* the reserve cells are hardly apparent. The large stainable balls of material are lacking and the few cells present are smaller in size, ranging from 19–29 μ with an average size of about 26 μ. In the few apparent cells, there is little evidence of either mucopolysaccharide material or calcium.

On the *fifth day,* these cells again reach a peak in abundance and size. With all stains and fixatives used, they are similar in every way to the three-day condition, with the exception of the fact that the mucopolysaccharide present stains only faintly with PAS, possibly indicating a decrease in concentration of the mucopolysaccharide material or the unavailability of its reactive groups to PAS. The cells range in size from 29–42 μ with an average of around 34 μ, are "mulberry-like" in appearance, and are again filled with calcium which shows up after appropriate fixation and staining.

By *six days*, the cells are fewer in number, somewhat smaller (range 17–27 μ, average 23 μ), but similar in staining properties to the fifth-day condition.

On the *seventh day* (end of Stage B) the cells are few in number but are somewhat larger in size (35–40 μ, average about 37 μ), and are detected after Bouin's fixation and toluidine blue staining and following A–F fixation and Mallory's, alizarin red S, purpurin, and Von Kossa's method, indicating again that these cells are loaded with calcium.

By *eight days* following molt (beginning of Stage C), the reserve cells are again not apparent.

It is, therefore, evident that the reserve cells even within the early postmolt period, Stage A and B, undergo, at daily intervals, cyclic peaks and declines in size and abundance, changes in structural appearance, and staining properties. The polysaccharide material which is distinctly evident up to the fifth day following molt is always diastase-fast and colors deep pink-purple with PAS but does not show gamma metachromasia with toluidine blue. Pearse (1953) has suggested that polysaccharide material staining in this way with PAS and frequently failing to show gamma metachromasia with toluidine blue probably indicates the presence of either a muco- or glycoprotein. As has been mentioned previously (Travis, 1955a) the reserve cells of late Stage C and Stage D animals stain with PAS in the same manner. This polysaccharide material probably represents reserve substances for the new skeleton and may, as was pointed out by Travis (1955a), during the premolt period represent breakdown products from the old skeleton.

None of the integumental tissues of *Panulirus*, unfortunately, were fixed for the detection of lipids. As was pointed out by Travis (1955a) the reserve cells within the connective tissue of the hepatopancreas contain much lipid and it would likewise be expected that the reserve cells of the sub-epidermal tissue also store it. Sewell (1955) has definitely shown that these reserve cells beneath the connective tissue of *Carcinus* do indeed store lipoprotein. These lipoprotein reserves reach a maximum in C_4 (late Stage C) and early D (D_1) and then begin to decrease, apparently as lipid content of the epidermis increases. These cells, as Sewell suggests, begin to disappear after the pre-exuvial layers of the skeleton are deposited and completely disappear by the end of Stage B. However, reserve cells of *Panulirus* become filled with calcium on the third, fifth, sixth, and seventh day following molt whereas on the first, second, and eighth day either no calcium or very little was apparent in the reserve cells. This would suggest to the present author cyclic peaks in calcium storage alternating with cyclic release to the epidermis as calcification of the skeleton occurs. The present author would expect this cyclic process (peaks and declines in size and abundance, changes in structural appearance, and calcium binding and release) to continue throughout early and middle Stage C, since calcification of the skeleton is not fully completed for at least three weeks following molt. If, therefore, the reserve cells arise from amoebocytes, as Sewell (1955) suggests, and possibly revert to amoebocytes after they have discharged their reserves, such a situation could clearly account for the cyclic peaks in size and abundance at varying daily intervals within a single stage of the molting cycle.

In this sense, the present author would be inclined to accept Sewell's suggestion that the reserve cells represent phases of activity of the amoebocyts with peaks not only before molting, as Sewell suggests, but following molt as well. The reserve cell cycle would then correspond more closely with the oenocyte cycle, being present throughout the molting cycle but reaching peaks at various phases of it. It might be pointed out that days in which reserve cells are scarcely apparent (2, 4, and 8 days following molt), large numbers of amoebocytes are apparent in the sub-epidermal connective tissue.

b. *The integument*

During the early postmolt period (Stage A and B) the post-exuvial layers of the skeleton are deposited. Of the post-exuvial layers of the outer integument, only the principal layer or calcified zone is progressively thickened during Stages A and B.

The amount of skeletal material, in total thickness, deposited per day, in the area of carapace from which sections of the integument were cut, during Stages A and B, varies from 14–72 μ with an average of around 38 μ. The thickness of the principal layer when fully formed varies from around 460–550 μ in animals used in this investigation (80–89 mm. carapace length). If one assumes that a constant amount of skeletal material is deposited daily in the principal layer, for example 38 μ, one can, by using this figure and the total thickness of the fully formed principal layer, roughly calculate the time in days when this layer is completed. By using these values, the calculated time at which the principal layer is completed would be around 15 days following molt. This time, however, is in actuality closer to 20 days following molt. Therefore, the membranous or non-calcified layer would not apparently begin to be deposited before the third week following molt (Stage C_3 of Drach, 1939). By the fourth week following molt (late Stage C or C_4 of Drach) the membranous layer is fully formed (Travis, 1955a).

The pigmented layer (toluidine blue-staining) shows the presence of a muco- or glycoprotein one through five days following molt but by the end of this period the tinctorial properties of this layer have decidedly changed. A deep purple rather than a pink-purple is given with this stain, indicating that the properties of the protein and closely associated chitin units of the pigmented zone have been changed by quinones. The newly formed principal layers initially show a green coloration with toluidine blue but shortly take on a pink coloration. The membranous layer, when fully formed, is light green in color with toluidine blue, possibly indicating differences in the organic nature or composition of this and the principal layers.

The inner integument, bordering the gill chamber, undergoes little if any thickening after the second day following molt. It attains a total thickness of approximately 30 μ, that of a late Stage C animal, by the second day following molt, indicating that the inner integument is completed during a period of three days preceding molt and two days following molt (Travis, 1955a).

As the post-exuvial layers are deposited during the early postmolt period (Stage A and B), concomitant hardening of these layers occurs by the deposition of mineral salts therein. It is evident from the analyses of small pieces of the area of softening (Travis, 1955b) that the most abundant mineral constituent in the exoskeleton is calcium. In order to determine what salts were deposited in the skeleton, further chemical analyses were carried out on the entire area of softening from late Stage C animals. The results of these analyses are indicated in Table I. From these results it is apparent that most of the calcium present in the skeleton is in the form of calcium carbonate. In order to determine whether this is deposited in the organic matrix of the skeleton as amorphous calcium carbonate or as crystalline aragonite or calcite, x-ray diffraction photographs (kindly made by Dr. C. Frondel, Department of Minerology, Harvard University), were taken of dry powder obtained from triturated pieces of the area of softening. These photographs (Fig. 3) indicate that calcium carbonate exists in the spiny lobster skeleton as calcite.

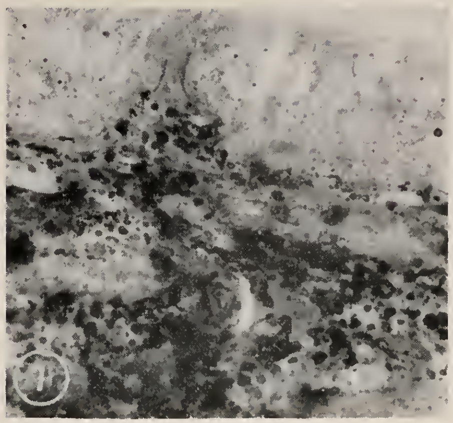
Since the basic organic components of the crustacean exoskeleton are chitin and protein, which are firmly associated with one another, Trim (1941), Stacy (1943) and Haworth (1946) regard the arthropod cuticle as a mucopolysaccharide because of the firm combination of carbohydrate-containing amino sugars (chitin) with the protein. Further, since calcium is the most abundant, if not the most important, mineral constituent within the crustacean skeleton, an emphasis will be placed on the abundance and distribution of glycogen, phosphatase, and calcium in the integumental tissues. These three constituents, among others, are of extreme importance in the development and calcification of the new skeleton.

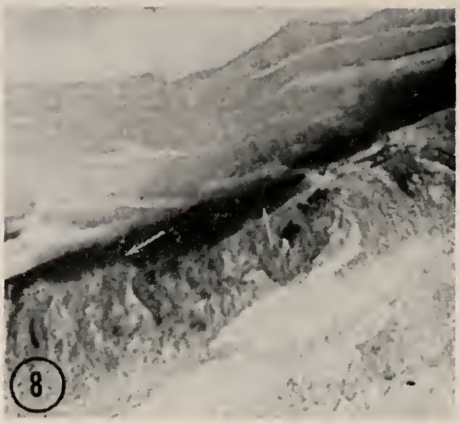
c. *Localization of glycogen, phosphatase and calcium*

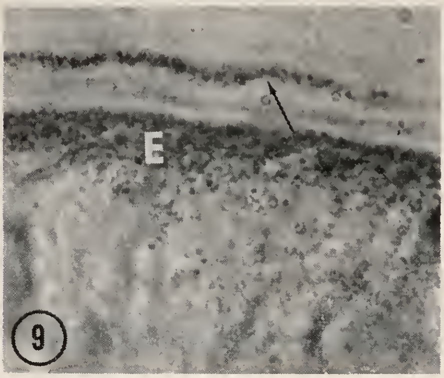
Glycogen: At *one day* following molt (Stage A), glycogen granules are scattered throughout the sub-epidermal connective tissue. Abundant amounts are concentrated at the bases of epidermal cells of the inner integument while the epidermal cells of the outer integument show little glycogen, which is localized in the proximal half of the cells (Fig. 4).

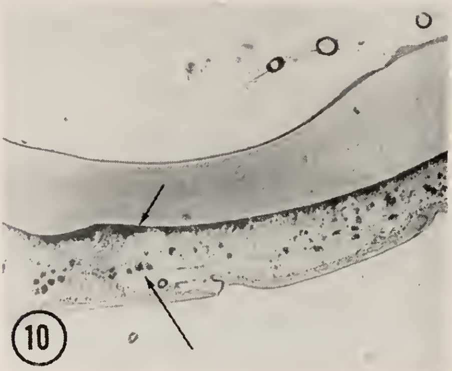
On the *second day* following molt, glycogen completely disappears from the inner epidermis (Fig. 5) and is not observed again in this tissue during the entire postmolt period. From two through four days following molt, little glycogen is apparent in the sub-epidermal connective tissues. Much heavier concentrations, on the other hand, are observed in the distal half of the outer epidermal cells (Fig. 6). By the *fifth day* glycogen accumulates in large amounts in the sub-epidermal tissues while the outer epidermis becomes almost depleted of it (Fig. 7). From the *sixth* through the *seventh day* most of the glycogen disappears from the sub-epidermal connective tissue and moves again to the outer epidermis where it is heavily concentrated in the distal portion of this tissue.

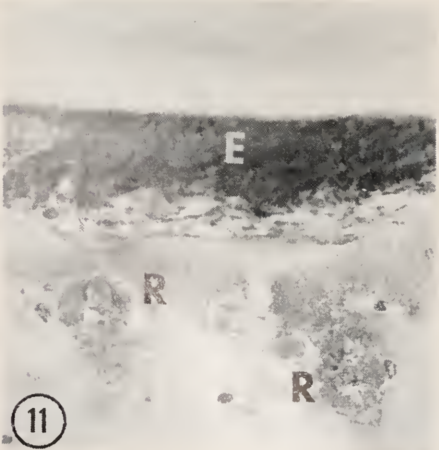
Phosphatase: Alkaline phosphatase becomes localized in the outer epidermis, being more heavily concentrated in the distal rather than the proximal half of the cells. Furthermore, the enzyme is heavily concentrated in the integument immediately above the epidermis (Fig. 8). It appears to be concentrated at this site in the region of the proximal portions of the innumerable pore canals. The localization of phosphatase in these sites is evident on the *first day following molt*, before calcification of the branchial integument begins, and remains in this localization throughout Stages A, B and very early C. In addition to its presence at these sites alkaline phosphatase is observed rather evenly distributed in the reserve cells during the entire period of observation. Control treated sections indicate that not only the enzyme but calcium as well are present in all of these sites from the second

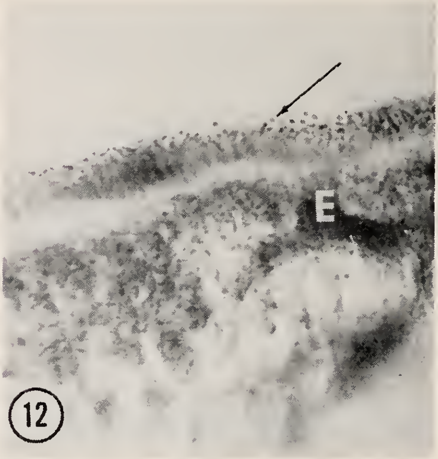












Figs. 7–12.

through the eighth day, although purpurin and alizarin red S did not show the presence of calcium within the reserve cells before the third day following molt. It might also be pointed out that the enzyme is likewise heavily concentrated around newly developing bristles.

Calcium: Calcification of the new outer integument of the branchiostegites begins on the second day following molt (Stage B). Before calcification begins, however, the epidermis begins to concentrate calcium in markedly evident amounts (detectable by purpurin, alizarin red S, Von Kossa's method, Gomori's (1941) method for alkaline phosphatase and confirmed by microincineration). Before discussing the calcification of the skeleton the author would like to point out that of these methods used for the detection of calcium, Von Kossa's method and Gomori's (1941) method for alkaline phosphatase are the most useful for showing actual stages in calcification of the integument. Furthermore, these two methods show calcium salts or complexes in granular form.

At *one day* following molt and one day before calcification of the integument begins, the distal ends of the epidermal cells begin to show calcium. No reserve cells of the integumental tissues, as will be recalled, bind calcium at this time.

By the *second day*, the distal portions of the epidermal cells are filled with calcium. The tissue spaces or sinuses at the base of the epidermis and the blood channels, sometimes observed between the epidermal cells, show the presence of calcium. Although only a few reserve cells are apparent, a few show scattered granular deposits of calcium. At this same period a narrow band of calcium granules appears in the new principal layer immediately distal to, but paralleling, the epidermis (Fig. 9). Likewise, immediately underlying the epicuticle and within the pigmented zone a heavy concentration of rows of granules is observed.

By the *third day* the epidermal cells as well as the reserve cells of the sub-epidermal connective tissue are completely filled with calcium (Figs. 10, 11) detected by all methods used for this purpose. The heaviest concentrations of calcium granules in the epidermis are observed in the distal half of the cell. Here they are observed to be extruded from the distal ends of the epidermal cells in two distinct ways (Travis, 1951a, 1951c). As the post-exuvial layers are deposited, masses of cal-

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FIGURE 7. At five days following molt glycogen has disappeared from the outer epidermis but is heavily concentrated by the sub-epidermal connective tissue. 760 ×.

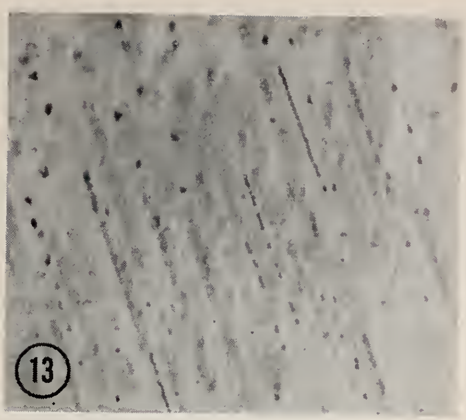
FIGURE 8. One day postmolt, before calcification begins in the branchial integument. Localization of alkaline phosphatase in the distal portion of the outer epidermis and in the integument immediately above the epidermis (region of the proximal portions of the innumerable pore canals, arrows). Phosphatase remains concentrated here through Stages A, B and early C. 420 ×.

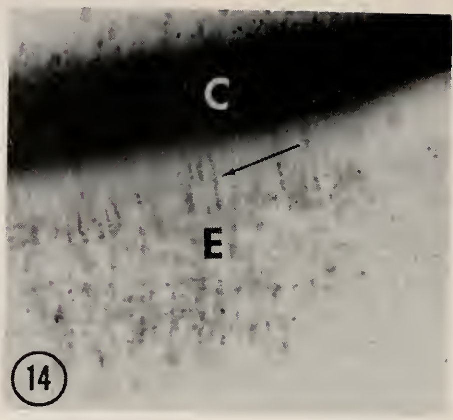
FIGURE 9. At two days postmolt calcification of the integument begins. Note that the distal portions of the outer epidermal cells (E) are filled with calcium and the narrow band of calcium granules (arrow) which appear in the newly forming principal layer immediately distal to, but paralleling, the epidermis. 2000 ×. Van Kossa's method.

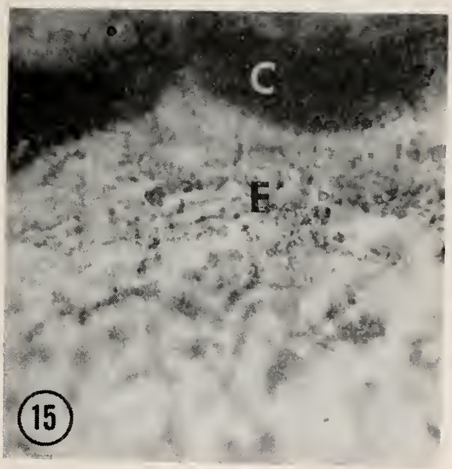
FIGURE 10. By the third day postmolt the epidermal cells (arrow) and the reserve cells (arrow) of the sub-epidermal connective tissue are filled with calcium. 90 ×.

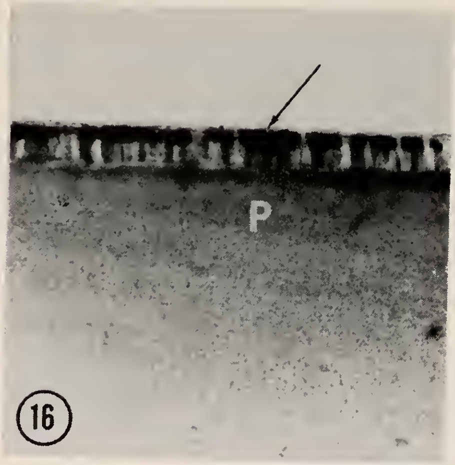
FIGURE 11. Higher magnification showing that the epidermal cells (E) and reserve cells (R) of sub-epidermal connective tissues, at three days postmolt, are filled with calcium. Note lumpy stainable balls of material in the reserve cells. 760 ×. Alizarin red S staining.

FIGURE 12. As the post-exuvial layers are deposited (three days following molt) masses of calcium granules are simultaneously extruded from the epidermal cells (E) to form narrow calcified bands paralleling the epidermis (arrow). 2000 ×. Van Kossa's method.









Figs. 13–18.

cium granules are simultaneously extruded to form narrow bands paralleling the epidermis (Fig. 12). In the pre-exuvial layers of the skeleton, deposited before molt, calcium granules are observed in uniform vertical rows. These rows of granules are particularly evident in the pigmented layer (Fig. 13) and can be seen to emanate from the epidermis (Fig. 14). The rows of granules correspond in location to the pore canals or vertical striae. Calcification of the pre-exuvial layers occurs after their formation. Hence, calcium has to be transported and deposited some distance from the epidermis. This transport, therefore, appears to occur through the pore canals.

From *four through seven days* calcium in the epidermis remains equal in amount to that observed on the third day.

Calcification continues to occur as the post-exuvial layers are deposited. As a consequence, the horizontal bands of granules paralleling the epidermis have thickened considerably by the end of Stage B (Fig. 15). By the *seventh day* portions of the inner integument are completely calcified. Calcification of the inner integument appears to occur in an identical fashion to that observed in the pre-exuvial layers of the outer integument.

2. *The hepatopancreas*

a. *Tissues*

The hepatopancreas, as discussed by Travis (1955a), is the major storage depot of organic and mineral reserves during Stage D and is, consequently, the major organ from which these reserves are mobilized when needed by other tissues during the postmolt period.

For five days following molt (Stage A and part of Stage B), the epithelial tissue of the hepatopancreatic tubules is predominantly of the absorbing type. This epithelial tissue consists of long tall columnar cells without large vacuoles and which may have either a central or basal nucleus (the so-called B_1 or R cells of Hirsch and Jacobs, 1928, 1930) as discussed by Travis (1955a). A few of the epithelial cells are of the secretory type (the vesicular or B_2 cells of Hirsch and Jacobs). These are enlarged swollen cells which enclose large vacuoles, some of

FIGURE 13. In the pre-exuvial layers of the skeleton, deposited before molt, calcium granules are observed in uniform vertical rows corresponding in location to the pore canals. 2000 ×. Von Kossa's method.

FIGURE 14. Note narrow vertical rows of calcium granules, corresponding in location to the pore canals (arrow) emanating from the epidermis (E). Also note the rather wide band of calcium granules (C) in a newly formed portion of the principal zone paralleling the epidermis. 2000 ×. Von Kossa's method.

FIGURE 15. Horizontal bands of calcium granules paralleling the epidermis (E) continue to be deposited as the post-exuvial layers are deposited and a thick calcified band (C) in the new principal layer is noted by the end of Stage B. 2000 ×. Von Kossa's method.

FIGURE 16. By seven days following molt (end of Stage B) the epicuticle (arrow) and most of the pigmented layer (P) appear to be fully calcified except in certain areas where this is not complete. 2000 ×. Von Kossa's method.

FIGURE 17. Note the heavy concentration of glycogen at the basal and distal ends of the absorption cells and at the periphery of the vacuoles of secretory cells in the tubular epithelium of the hepatopancreas. One day following molt (Stage A). 200 ×.

FIGURE 18. At seven days following molt (end of Stage B) the tubular epithelium of the hepatopancreas is virtually devoid of glycogen. 200 ×.

which contain stainable material. These cells (Travis, 1955a) undergo apocrine breakdown in *Panulirus*. Their vacuolar contents plus adjacent cytoplasm are discharged into the lumen of the hepatopancreatic tubules, leaving only the basal region and nucleus of the cell intact. However, none of these observed secretory cells, during this period of five days following molt, show any sign of apocrine breakdown. This would be expected because the animals are still undergoing a period of inanition (Travis, 1954, 1955a, 1955b). Stainable material in the large vacuoles of the secretory cells frequently indicates the presence of lipid and mucopolysaccharide.

By the sixth and seventh day following molt, the predominant cell types observed in the epithelial tissue of the tubules are the secretory cells. On the seventh day there is much evidence of apocrine secretion within these cells. In general, most animals begin to feed again on the seventh day. This would not only account for the numerous secretory cells but also for the apocrine breakdown of many of these cells.

The large oval reserve cells are likewise apparent in the connective tissue between the individual tubules of the hepatopancreas. They similarly show, as do the reserve cells of the integumental tissues, the presence of phosphatase, mucopolysaccharide and calcium. Much lipid is also present during Stage A and B. From the fifth through the seventh day the reserve cells of the hepatopancreas show the presence of large vacuoles (Fig. 2), some of which contain flaky or granular-like stainable material while others appear to be clear. This is possibly correlated with a decrease in mucopolysaccharide, lipid, and calcium content in these cells. The reserve cells of the hepatopancreas, like those of the integumental tissues, undergo cyclic peaks and declines in size and abundance, changes in structural appearance, and the storing and apparent release of reserves as indicated below.

During the *intermolt period* (late Stage C) they are numerous and large, ranging in size from 17–38 μ with an average size of 30 μ. They show markedly evident amounts of mucopolysaccharide. A similar situation prevails during the *premolt period*. The cells range in size from 17–38 μ with an average size of 32 μ and show large stainable spheres of material. At both of these stages much mucopolysaccharide is present. Calcium is likewise apparent in large amounts in the hepatopancreatic reserve cells, while the integumental tissue reserve cells are devoid of it during these periods.

By the *first day* following molt, there are few reserve cells present in the hepatopancreas and these have undergone a general decrease in size (8–29 μ, average size 16 μ), a situation opposite to that observed in the integumental tissues. Of the reserves, however, little calcium seems to be apparent and there is no evidence of spheres of material found within them. On the *second day* (beginning of Stage B) the cells remain few in number but have increased in size (range 24–38 μ, average size 31 μ), again a situation opposite to that observed in the integumental tissues, although no large spheres of material are evident. They again show the presence of small amounts of calcium. At *three days*, as in the integumental tissues, the reserve cells become numerous and remain large (range 24–48 μ, average size 30 μ). They take on a distinct "mulberry" appearance and show the presence of mucopolysaccharide and markedly evident amounts of calcium. A similar situation prevails on the *fourth day*. The cells in the hepatopancreas remain numerous and large (range 26–43 μ, average size 35 μ) and likewise show abundant mucopolysaccharide and calcium. By the *fifth day* few cells are apparent and they are slightly smaller in size (21–48 μ, average size 28 μ). These cells become highly vacuolated, although some balls of material are present within the vacuoles, and show only a slightly pink coloration with PAS, a condition similar to that observed in the reserve cells of the integumental tissues. Little calcium is apparent. Except for the increase in number and slight increase in size (29–38 μ, average size 32 μ) the reserve cells on the *sixth day* remain similar to the *fifth day* condition though they contain large single vacuoles with no stainable balls of material. They remain numerous on the *seventh day* but the average size is slightly smaller (24–32 μ, average size 29 μ). Except for these changes they are similar to the *fifth day* condition with respect to reserves. It should be added that at all stages of the observation period, phosphatase and lipid are present.

Since different groups of animals were used for the study of hepatopancreatic and integumental tissues (fixed in different years), it is difficult to determine whether or not the cycles may be slightly out of phase at the sites of observation. The information at hand (Sewell, 1955; Travis, 1955a) strongly suggests that much more information is needed on these highly interesting and obviously important reserve cells.

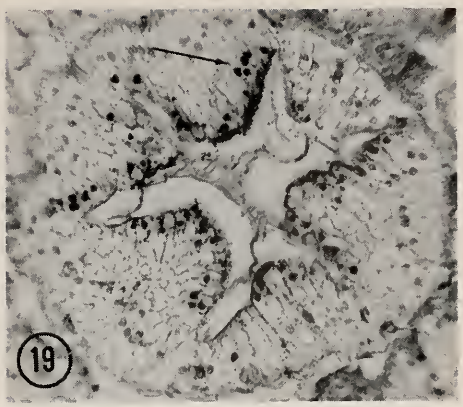
b. *Localization of glycogen, phosphatase, calcium and lipid*

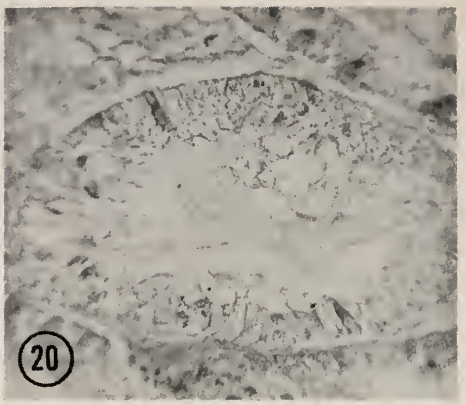
Glycogen: Although the concentration of glycogen within the tubular tissue on the first day following molt (Fig. 17) would compare with the premolt condition (see Figure 30; Travis, 1955a), there is a progressive decrease in abundance from the first through the seventh day. A decidedly marked decrease occurs by the sixth day and by the seventh day hardly a single granule of free glycogen can be detected within the tubular tissue (Fig. 18). During Stages A and B, glycogen is more heavily distributed in the distal and basal ends of the absorption cells. When mature secretory cells are observed during the early phase of Stage B, glycogen granules are localized at the periphery of the vacuoles and are sometimes observed within the lumen of the tubules.

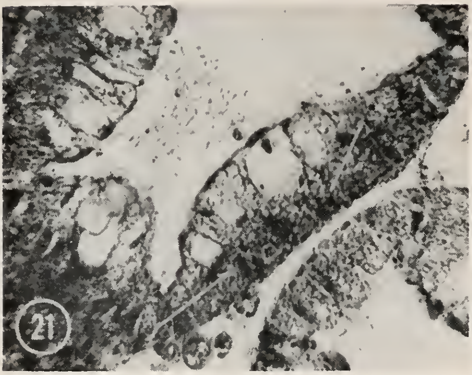
The disappearance of glycogen from the hepatopancreas by the seventh day might be expected because of the need of this constituent in the synthesis of the new skeleton. The integumental tissues, among others, therefore, accumulate and use large amounts of glycogen at the expense of the hepatopancreas. This is evident in both the premolt and postmolt period.

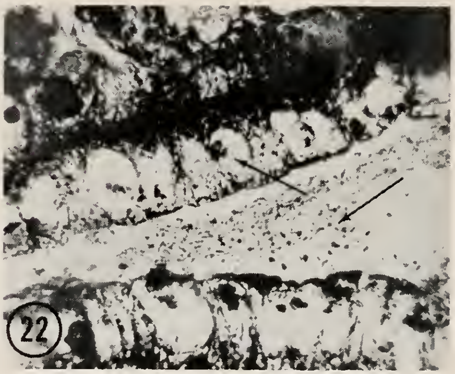
Phosphatase: During the early postmolt period alkaline phosphatase is almost absent from the striated borders of the tubular tissue but remains localized around the calcospherites (Fig. 19), which disappear progressively as calcification of the skeleton occurs. From the sixth through the seventh day when many large secretory cells are present, the enzyme is localized around the periphery of small and large secretory vacuoles. It is at all times present in the reserve cells.

Calcium phosphate: As in the premolt animals, the tubular epithelium of the postmolt animal is marked by the presence of innumerable calcospherites in the apical ends of the absorbing cells, very few being apparent in the small number of secretory cells. In the postmolt animal, however, these calcospherites disappear progressively as the skeleton is calcified. They have markedly decreased by the fifth day and by the seventh day (Fig. 20) hardly a single calcospherite can be de-









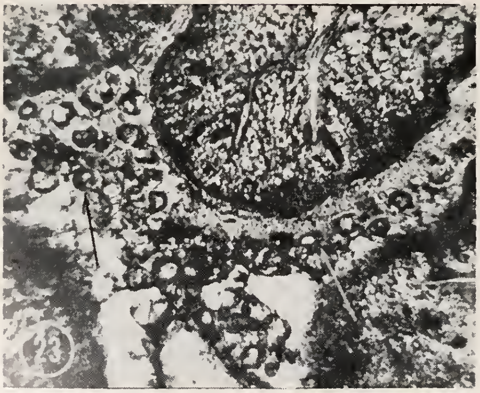


FIGURE 19. The tubular epithelium of the hepatopancreas at one day following molt (Stage A). Note alkaline phosphatase localized around innumerable calcospherites (arrow) at the apical ends of absorbing cells. 200 ×.

FIGURE 20. At seven days following molt, note that there is almost a complete absence of calcospherites and the enzyme alkaline phosphatase in the tubular epithelium of the hepatopancreas, a situation that occurs progressively as the integument is calcified. 160 ×.

tected. The complete disappearance of the calcospherites by the seventh day would be expected in *Panulirus* because these animals have to rely completely on this stored phosphate, stockpiled during the two-week premolt inanition period, as a source of phosphate for incorporation into the new skeleton. Since, as was pointed out previously (Travis, 1954, 1955a, 1955b), phosphate is obtained primarily from food, the stockpile of stored phosphate in the hepatopancreas would readily be depleted by the sixth or seventh day of starvation following molt. From low blood values following molt (Travis, 1955b) it is evident that the normal intermolt blood concentration within the body would not be replenished for at least three or four weeks following molt. Although the reserve cells of the hepatopancreas show the presence of calcium from one through seven days following molt, from the fifth through the seventh day little is apparent.

Lipid: For the entire observation period (Stages A and B) droplets of lipid are found throughout the epithelial tissue of the hepatopancreatic tubules. There would appear, by the sixth and seventh day, to be a decrease over that observed in the premolt animal, although much is still apparent. When secretory cells are apparent in great numbers (6 and 7 days) lipid droplets are frequently observed within the vacuoles (Figs. 21, 22). On the seventh day, when apocrine breakdown is evident, and when some of the animals begin to feed a little, lipid material becomes quite apparent within the lumen of the tubules, a condition which would be correlated with extracellular digestion of this constituent, as Van Weel (1955) has shown.

The reserve cells contain considerable quantities of lipid for the entire postmolt observation period (Fig. 23). There does appear to be a decrease over that observed during the intermolt (late Stage C) and the premolt period. Because of the presence of considerable quantities of lipid within the hepatopancreas at this time, a histochemical or a qualitative difference in amount is difficult, with certainty, to detect.

Discussion

The continued accretionary growth and hardening of the post-exuvial layers of the skeleton imposes upon the epidermis two major tasks, namely the synthesis and elaboration of the organic matrix and the simultaneous or accompanying elaboration of constituents for hardening the skeleton which may or may not alter the properties of the basic organic components, chitin and protein. The complexities of these two functions cannot be over-emphasized. Although the epidermis takes the lead in the performance of these tasks, the importance of other tissues, such as the hepatopancreatic and subepidermal tissues, cannot be under-estimated.

As the principal layer of the skeleton in *Panulirus* is deposited during the early postmolt period of observation (Stages A and B), the outer epidermis shows, tinctorially, that considerable amounts of a glyco- or mucoprotein are concentrated or

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Figure 21. The distribution of lipid droplets within secretory cells of the hepatopancreas (arrows). The secretory cells become apparent in great numbers at six and seven days following molt. 200 ×.

Figure 22. Seven days following molt. Note lipid droplets (arrow) within the vacuoles of the secretory cells and within the lumen of the tubules (arrow). 200 ×.

Figure 23. Note that considerable quantities of lipid are bound by the reserve cells (arrows) of the hepatopancreas. 100 ×.

synthesized by this tissue. Similarly, during Stage A and most of B, the pigmented layer, one of the two pre-exuvial layers formed before molt, shows the presence of this same mucopolysaccharide. By the fifth day the pigmented layer no longer indicates a positive reaction for this constituent. There is a tinctorial change from gamma metachromasia (pink-purple) to beta metachromasia (deep purple) with toluidine blue, which indicates that the properties of the basic organic components, chitin and protein, have been altered. Possibly this is caused by considerable impregnation with calcium salts at this time or, more certainly, by quinones which form cross-linkages with the native protein phase of the cuticle (Pryor, 1940). The net result of this combination is the formation of a highly stable and insoluble product. As Krishnan (1951) pointed out, the tanning by quinones of the pigmented layer occurs in *Carcinus maenas* shortly following molt and this is followed by pigmentation at a slightly later period. It is, therefore, possible that both of these related processes are completed by the fifth day in *Panulirus*, thus causing this change in tinctorial properties.

The presence of muco- or glycoprotein in the epidermis for the entire postmolt observation period (Stages A and B) is doubtlessly related to the secretion and development of the principal layer, which is the only post-exuvial layer deposited during this period. This layer, like the pre-exuvial pigmented layer and post-exuvial membranous layer (formed and completed from the third through the fourth week following molt in *Panulirus*), consists as in insects of the basic organic components, chitin and protein, which are closely associated with one another. The firm combination of these two organic constituents has led Trim (1941), Stacy (1943) and Haworth (1946) to regard the arthropod cuticle as a mucopolysaccharide because of the firm combination of the carbohydrate-containing amino sugars (chitin) with the protein. Richards (1951) has pointed out that the consideration of the arthropod cuticle as a double set of layers (the outer set being composed of lipoprotein and the inner set being composed of glyco- or mucoproteins) is advantageous. This consideration emphasizes that the major cuticular components seem to be formed and secreted as conjugated proteins and not as separate components.

The principal layer in *Panulirus* always shows, tinctorially, the presence of muco- or glycoproteins with the exception of the fact that immediately after each layer is deposited a green, rather than a pink, coloration with toluidine blue occurs. This suggests that the reactive groups are slightly altered following their immediate formation. Although the early stages in the formation of the membranous layer were not followed, the completed layer, in contrast to the principal layer, shows a green rather than a pink coloration, which indicates that this layer is, in some way, different from the principal layer. This is further revealed by the fact that calcium salts are never bound in this layer.

During the deposition of the principal layer of the outer integument glycogen accumulates in large amounts in the epidermis. There is a periodic shift of glycogen from the sub-epidermal tissues to the outer epidermis (Travis, 1951a, 1951c, 1955a) and in turn a shift from the hepatopancreatic tissues to these integumental tissues (see Observations). During the postmolt period of accumulation and utilization of glycogen by the integumental tissues (Stages A and B), hepatopancreatic glycogen progressively disappears. Although it is abundant in the tubular tissue on the first day following molt, there is a marked decrease in glycogen by the fifth day and hardly a single granule of free glycogen is present by the seventh day. The rhythmical accumulation by, and disappearance of, glycogen from the sub-epidermal tissues and similarly its accumulation by the epidermal cells and disappearance from the sub-epidermal tissues suggests a rhythmical cycle of accumulation and utilization in the epidermis, at the expense of sub-epidermal tissue glycogen. Likewise, the rhythmical accumulation of glycogen by the sub-epidermal tissue and progressive disappearance from the hepatopancreas similarly suggests that there are marked cycles of accumulation and utilization. These cycles of accumulation and utilization stem from the epidermis, which takes the lead in the elaboration of the post-exuvial layers of the integument, but also involves the hepatopancreas, which serves as the major storage organ from which such reserves can be mobilized and upon which the epidermis is ultimately dependent for the successful completion of its tasks. Since feeding begins on the seventh day following molt in the summer months, a constant supply of glycogen would be available to the epidermis until the integument is completed (late Stage C), but is not stockpiled in the hepatopancreas again until Stage D. The stockpiling of glycogen during this period of inanition would suggest strongly that the source of this constituent is from the large quantities of lipid reserves, likewise present in the hepatopancreas at this time. By the conversion of some lipid, through its glycerol moiety, to carbohydrate, the latter being stored as glycogen, the peak glycogen concentration could be achieved during Stage D. As was pointed out by Travis (1955a), evidence suggests (Renaud, 1949) that during periods of inanition (Stages D, A and B), lipids likewise serve as a major source of energy by playing a principal role in oxidative metabolism.

The periodic accumulation and utilization of glycogen by the epidermis as the post-exuvial layers are deposited suggests that glycogen is a necessary precursor for chitin formation. This possibility, as discussed by Travis (1955a), has been suggested by Verne (1924, 1926), Mataczyńska-Suchcitz (1948), Renaud (1949), Travis (1951a, 1955a) and Schwabe *et al.* (1952). Glycogen may likewise serve as a ready energy-source for the synthesis and elaboration of the organic constituents of the integument. This possibility has been suggested by Bradfield (1951). He found an abundance of glycogen in regenerating epidermis of the vertebrates. As the outermost cells keratinized, glycogen disappeared. He attributed this disappearance to the utilization of glycogen for the supply of energy in keratin synthesis. Glycogen may further serve indirectly as added substrate for phosphatase action after its hydrolysis and phosphorylation by phosphorylases. In this way, it has been postulated as one of the necessary mechanisms in calcification of bone and teeth of vertebrates (Robison and Soames, 1924; Harris, 1932; Glock, 1940; Horowitz, 1942; Engel, 1948; Marks and Shorr, 1950 and others). It is more likely, however, that glycogen participates in all of these functions and possibly others that have not been mentioned.

During the entire postmolt observation period (Stage A and D), alkaline phosphatase is heavily concentrated in the distal ends of the outer epidermal cells and is observed in the integument immediately distal to but paralleling the epidermis. The localization of the enzyme in this latter site is distinctly apparent by the first day following molt before any calcification begins in the branchial region of the integument. Krugler and Birkner (1948) noted a similar localization of the enzyme in the integument of the crayfish during premolt. In *Panulirus* this is a strategic location for the enzyme during the postmolt period because it is in a region of high activity as the deposition and hardening of the post-exuvial layers occur. Further, it would appear that the enzyme may be specifically localized in the proximal portions of the pore canals. Because of its heavy concentration along this entire region of the integument, however, its specific localization in the pore canals is difficult to determine with certainty. The enzyme is likewise heavily concentrated in the integument around newly developing bristles. In the sub-epidermal tissues it is observed in the reserve cells.

In the hepatopancreas the most marked localization of the enzyme is seen around the innumerable calcospherites in the distal portions of the absorption cells. The enzyme is observed in the striated borders of these cells and on the sixth and seventh day, when a predominance of secretory cells is evident, it is observed at the periphery of small and large vacuoles. The reserve cells within the blood or tissue spaces between the tubules of the hepatopancreas likewise show the presence of the enzyme.

As in the premolt animal, phosphatase is localized around the calcospherites in the absorption cells of the hepatopancreas and since these disappear progressively as calcification of the post-exuvial layers occurs, it is possible that the enzyme participates in the mobilization of this reserve for transfer to the integument. It may do so by dephosphorylating, in some way, the precipitated complex. In this role, it would be serving in resorption at this site and could at the same time be involved in mediating the synthesis of other phosphoric esters to be conveyed via the blood from the hepatopancreas to the integumental tissues. That this indeed may be an important function of phosphatase in bone resorption has been suggested by McLean and Urist (1955). Further, its localization around the periphery of small and large vacuoles on the sixth and seventh day would suggest that the enzyme is possibly involved, in some way, with the synthesis of secretory products or the transfer of these products from the adjacent cytoplasm into the secretory vacuoles. Phosphatase localization at the striated borders of the absorption, as well as the secretory, cells would suggest that when these cells are active the enzyme would likewise serve the function of participating in transfer reactions by producing molecules which enter or leave the cells more readily. Such a function has been suggested by Moog (1946).

The concentration of the enzyme at the distal ends of the outer epidermal cells and in the integument immediately distal to, but paralleling, the epidermis suggests its extremely important functions in the deposition and hardening of the post-exuvial layers. The periodic accumulation and utilization of glycogen by the epidermis, as was pointed out earlier, would suggest that possibly this constituent, glycogen, is used as a precursor in chitin formation. If this is so, and if the synthesis of chitin occurs, as Renaud (1949) suggested, by the hydrolysis of glycogen and dephosphorylation of glucose phosphate to glucose, this step being followed by subsequent steps to yield chitin, phosphatase would play an important role in this chain of events by its dephosphorylation of glucose phosphate to glucose, a possible starting point for chitin formation. Likewise, if glycogen were used as an energy-source for the synthesis and elaboration of the organic matrix, phosphatase would be intimately involved in these reactions. Glycogen, as suggested earlier, could serve indirectly as added substrate for phosphatase action. The distribution of phosphatase and mucopolysaccharide in the epidermis and its distribution in the region of the newly forming post-exuvial layers of the integument, immediately above the epidermis, suggests that it may play a very important part in the formation of the ground substance (mucopolysaccharide) of the post-exuvial layers. Furthermore, the enzyme is thought to play an important role in the manufacture of fibrous proteins, thus participating in the formation of the ground substance of bone (McLean and Urist, 1955). Moog and Wenger (1952), however, have suggested that since the enzyme and mucopolysaccharide are frequently found together in fibrous structures, the mucopolysaccharide constitutes part of a cytoskeletal mechanism to which the enzyme is bound.

The appearance of alkaline phosphatase in the integument immediately distal to and paralleling the epidermis one day before calcification begins, likewise suggests to the author that the enzyme is intimately involved in calcification of the integument. In such a localization it could provide a mechanism for the production of a local high concentration of phosphate ions. In the presence of calcium ions, transferred across the cell membranes of the epidermis, phosphate could then unite to form the calcium salt, calcium phosphate, which constitutes about 3% of the total mineral salt of the integument (Table I). The almost certain presence of phosphorylases at these sites, although not specifically determined, would likewise be expected to be important in the calcification of the integument, by synthesizing potential substrates for phosphatase action in zones of calcification.

TABLE I

Analyses to indicate the amount of mineral and organic matter in the entire area of softening of a late Stage C animal

Substance analyzed	Per cent present in the area of softening	Calculated % of salts present	
CaO	24.64	$CaCO_3$	42.39
MgO	1.98	$MgCO_3$	4.04
P_2O_5	1.40	$Ca_3(PO_4)_2$	3.05
CO_2	21.39	Carbonates unaccounted for	2.22
Per cent mineral as Ca, P, and Mg oxides	28.02		
Per cent organic matter	71.98		

Hardening of the crustacean skeleton occurs by quinone tanning and calcification. Hardening by quinones is a result of the oxidation of polyphenols to quinones, which form cross linkages with the native protein of the cuticle. The net result of this combination is a highly stable and highly insoluble product. Of the pre-exuvial layers, the epicuticle of *Carcinus maenas* is hardened by quinones shortly after its formation, whereas subsequent hardening of the pigmented layer occurs soon after molt (Krishnan, 1951). As was suggested in a previous section of the discussion, changes in tinctorial properties of the pigmented layer suggest that the process of hardening by quinones is complete by the fifth day following molt. Quinone tanning, although the primary cause of hardening in the exoskeleton of insects, plays a much smaller role in Crustacea (Dennell, 1947), calcification being the major cause of hardening.

Calcification begins on the second day following molt in *Panulirus* and occurs thereafter simultaneously with or immediately accompanying the elaboration of layers of the principal zone. Further, the additional task posed to the epidermis is that of calcifying the *pre-exuvial layers;* calcification, in this case, is of course a process that is accomplished long after their formation but during the same time at which the post-exuvial layers are being calcified.

In the distal region of the epidermal cells, where calcium becomes most heavily concentrated, extrusion of calcium from this tissue occurs in two distinct ways (Travis, 1951a, 1951c). As the post-exuvial layers are deposited, masses of calcium granules are simultaneously extruded, thus forming narrow bands paralleling the epidermis. In the pre-exuvial layers, on the other hand, calcium granules are observed in uniform vertical rows. These rows, as pointed out earlier, are particularly evident in the pigmented layer, and are likewise observed to emanate from the epidermis. They correspond in location to the pore canals or vertical striae, protoplasmic extensions of the epidermis. Thus, in the case of the pre-exuvial layers, calcification occurs after their formation. Hence, calcium must be transported and deposited some distance from the newly forming pre-exuvial layers. This transport occurs through the pore canals, thus enabling the epidermis to act at these distant sites.

Calcification of the integument continues and is almost entirely completed by the seventh day (end of Stage B) in the epicuticle and most of the pigmented layer.

While calcification of the integument occurs, the reserve cells in the subepidermal tissues undergo what appear to be cyclic peaks in calcium storage, possibly alternating with cyclic release to the epidermis. If calcium in these cells is used periodically by the epidermis, which it probably is, the reserve cells could serve as reservoirs for providing additional calcium during periods of concentration by the epidermis. At no time during Stage A and B, however, are the epidermal cells depleted of calcium. This might be expected because the concentration of calcium in the blood (Travis, 1951b, 1955b) is sufficiently high to provide a continued supply of this element to the epidermis. Previous reference to the reserve cells has already been made as to their cyclic peaks and declines in abundance and size, change in structural appearance and in the binding and release of reserves, other than calcium (see Observations) and will not therefore be discussed in this section.

As calcification of the integument occurs there is a progressive decrease in number of calcospherites—spherules of calcium phosphate—present in the absorption cells of the hepatopancreas. Though abundant on the first day following molt they progressively decrease in number as calcification of the new integument occurs and by the seventh day hardly a single calcospherite is to be detected. The calcospherites, premolt storage depots of reserve phosphate from the old skeleton (Travis, 1955a), represent a major source, therefore, from which phosphate can be mobilized for hardening of the new skeleton during Stage B, a time at which the animals do not feed. Since the spiny lobster obtains most of its phosphorus from food and since the animals do not feed for two weeks before molt, when resorbed phosphorus from the old skeleton is being stockpiled in the hepatopancreas (Travis, 1955a), one would expect a depletion of this mineral reserve during the first week following molt as calcification of the new integument occurs. This depletion is also evidenced by the low blood-phosphorus levels following molt (Travis, 1955b).

It is interesting that the reserve cells of the hepatopancreas show the presence of calcium from one through seven days following molt but from the fifth through the seventh day little is apparent. The fifth through the seventh day is a period when these cells become vacuolated, which may be correlated with the apparent decrease in mucopolysaccharide, lipid and calcium. It is possible that as the calcium phosphate is mobilized from the calcospherites it is immediately transferred from the absorption cells to the reserve cells and from these to carriers in the blood, possibly organic acids of oxidative and glycolytic metabolism. The evidence at hand, however, is not sufficient at this time to determine whether there is an actual movement of the reserve cells from the hepatopancreatic tissues to the integumental tissues.

Little has been said about the development and hardening of the inner integument during Stages A and B. It will be recalled that glycogen is observed in abundance at the bases of the epidermal cells of the inner integument on the first day following molt (Stage A) but completely disappears from the inner epidermis by the second day, not to be observed again at this site for the entire observation period. Similarly, no further thickening of this integument occurs after the second day following molt, indicating that the development of the inner integument of the branchiostegites is completed during a period of three days preceding molt and two days following molt (Travis, 1955a). This does not mean, however, that hardening by calcification is completed at this time. Calcification of the inner integument, as in the pre-exuvial layers of the outer integument, occurs after its formation. By the fourth day the uniformly staining endocuticle of the inner integument has begun to calcify and by the seventh day portions of it have completely calcified. Calcification in the inner integument occurs in the same fashion as that observed in the pre-exuvial layers of the outer integument, *i.e.,* via the port canals.

The two major tasks which must be achieved by the epidermis, namely, the synthesis and elaboration of an organic matrix and the simultaneous or accompanying elaboration of calcium salts for hardening of the newly developing skeleton, are not completely accomplished before the fourth week following molt during the summer months. Calcification is probably completed by the third week following molt, a time at which the membranous or non-calcified layer begins to be formed. This layer is not fully completed before the fourth week following molt (late Stage C or C_4 of Drach, 1939). Approximately three weeks following the completion of the integument the epidermis is again confronted with the preparation for growth in size of the animal (Travis, 1955a).

The cells of the epidermis, like the osteoblasts of bone, synthesize and elaborate the organic matrix of the skeleton and, unlike the osteoblasts of bone, they actually concentrate and secrete the mineral constituents, principally calcium, which are precipitated in the matrix. Furthermore, the epidermis, like the osteoclasts of bone, participates intimately in processes of resorption of the integument. It elaborates the proteinases and chitinases which break down the organic matrix. It likewise resorbs these organic breakdown products along with the mineral constituents and also participates in their transfer across its cell membranes to the blood for further handling (Travis, 1955b).

Following molt the transfer of calcium ions from the blood across the cell membranes of the epidermis and the concentration of calcium by this tissue is truly remarkable. Within the epidermal cells, the calcium is doubtlessly immobilized ionically by the binding capacity of weakly acidic groups of protein, succinate, lactate, bicarbonate, phosphate, citrate or by other anionic groups. On release of calcium to the exterior of the cell, an alteration in the binding capacity of anionic groups is necessary. After release from the epidermis, calcium is precipitated as salts, by various mechanisms, in the organic matrix of the integument.

The distribution of alkaline phosphatase in the distal portion of the epidermis and particularly in the integument immediately distal to and paralleling the epidermis suggests that this enzyme would provide a mechanism for the local high concentration of phosphate ions, which in the presence of some of the calcium released from the epidermis could account for the precipitation of the 3% calcium phosphate of the integument (Table I).

Calcium carbonate, however, is the principal salt of the spiny lobster skeleton, and Crustacea in general, and constitutes approximately 42% of the total mineral deposited in the skeleton of *Panulirus*. It is, therefore, of interest to point out a related and possibly important enzyme involved in calcification of the skeleton. This is the enzyme, carbonic anhydrase. Sabotka and Kann (1941) found that this enzyme was not present in the gills of *Panulirus argus*. Because of this, they suggested that elimination of CO_2 is not confined to the gills, but that the bicarbonate formed may be eliminated in the skeleton by precipitation as $CaCO_3$. Maluf (1940) found that considerable quantities of carbonic anhydrase were present in the epidermis and skeleton of the crayfish, *Cambarus clarkii* and the American lobster, *Homarus americanus*. The fact that alkaline phosphatase is found in the distal ends of the epidermal cells and integument immediately above and paralleling the epidermis would suggest that the hydrogen ion concentration is low and the pH is on the alkaline side (between 8–10). At this pH, dissociation of bicarbonate into $CO_3^=$ and H^+ would be expected. In the presence of calcium ions released from the epidermis, the precipitation of calcium carbonate could occur. Thus, again the mechanism and conditions exist for the production of local high concentrations of bicarbonate and carbonate ions in the epidermis and integument of Crustacea.

The manner in which calcium carbonate is precipitated, *i.e.*, as calcite rather than aragonite or amorphous calcium carbonate, is undoubtedly determined by conditions inherent in the organic matrix which favor calcite precipitation. Prenant (1927), however, pointed out that the condition determining the state of calcium carbonate precipitation is the proportion of phosphates to carbonates, as indicated by the P_2O_5/CO_2 ratio. If the ratio is more than 0.015, calcium carbonate is deposited in amorphous form. If the ratio is 0.105 or less, the calcium is deposited in crystalline form. The P_2O_5/CO_2 ratio in *Panulirus* is 0.0657. Calcium carbonate in *Panulirus* is precipitated as calcite, which is consistent with the idea proposed by Prenant. This would not mean, however, that conditions in the organic matrix do not favor calcite, rather than aragonite, precipitation.

That the calcification mechanism in *Panulirus* may be influenced by the presence of organic acids such as lactic, succinic, and citric acid at the sites of calcification has yet to be investigated. These acids have a marked propensity for forming weakly ionized salts with calcium. It is highly likely, therefore, that, as in the calcification of bone, citric acid plays an important role in the calcification mechanism of Crustacea. It is known from the work of Dickens (1941) that more than 70% of the citric acid of the human body is in the skeleton and that as much as 1% of the fresh weight of bone may be accounted for as citrate. It is not definitely known whether citrate is present as an ion or precipitated as a calcium citrate complex. Bones examined for enzymes of the citric acid cycle have shown that by comparison with other tissues, such as the kidney or liver, citrogenase and aconitase activities are much greater than those of isocitric dehydrogenase (McLean and Urist, 1955). As these authors point out, the mechanism for the production of local high concentrations of citric acid exists in bone. Other than the work of Thunberg (1949), however, as quoted from Thunberg (1953) and Steinhardt (1946), nothing has been done with the role of citric acid in the calcification process of the Crustacea. Thunberg found that at least 0.8% of the gastroliths of a European crayfish was constituted of citric acid. This would suggest strongly that citric acid is involved, as in bone, in some way in the calcification process. Steinhardt (1946) has further pointed out that in structures such as bone and gastroliths, where high concentrations of citric acid are found, the phosphorus and calcium content is also high. In such cases, citrate probably exists in a complex form in which calcium, phosphoric and citric acid enter.

It is highly likely, therefore, that within the integument of Crustacea, as in the gastroliths of the crayfish, the mechanism for the production of local high concentrations of citric acid exists. If so, the citrate produced, having a marked propensity for forming weakly ionized salts with certain cations such as calcium, could combine with calcium released from the epidermis and could enter into the mineral complex of calcium salts of the integument. If this is the case, increases and decreases of citrate formed enzymatically within tissues may be one of the regulators of ionic calcium activity. Furthermore, in normal metabolism, the normal activity of tissues may be controlled, in part, by the interaction of ionic calcium with citrate reached via the tricarboxylic cycle. Such a possibility has been suggested for the mammal by Peters (1950).

It is apparent from the foregoing discussion that the continued growth of the skeleton in an accretionary manner and the hardening of it by calcification, two of the major tasks confronting the crustacean from Stage A to late Stage C, are indeed complex. Although these extraordinary duties are put to the epidermis, which takes the lead in the performance of them, the importance of the other tissues, namely the hepatopancreatic and sub-epidermal tissues, should not be under-estimated.

Summary

1. During the early postmolt period (Stages A and B) as rapid accretionary growth and calcification of the skeleton are occurring, changes are observed in the hepatopancreas and integumental tissues.

2. As the principal layer of the skeleton in *Panulirus* is deposited during Stage A and B, the outer epidermis concentrates or synthesizes a considerable amount of glyco- or mucoprotein, which is probably related to and involved in the secretion and development of this layer. Similarly, the pigmented layer, one of the pre-exuvial layers formed before molt, shows the presence of this same mucopolysaccharide. Near the end of Stage B, however, the properties of the basic organic components in the pigmented layer have been altered, possibly by considerable impregnation with calcium salts or by quinones.

3. During the deposition of the principal layer of the outer integument, glycogen accumulates in large amounts in the epidermis. There is a periodic shift of glycogen from the sub-epidermal tissues to the outer epidermis and in turn a shift from the hepatopancreatic tissues to the integumental tissues. During this period

of accumulation and utilization by the integumental tissues (Stages A and B), hepatopancreatic glycogen progressively disappears and by the end of Stage B none remains.

4. The possibility of lipid conversion to carbohydrate, and the storing of this as glycogen in the hepatopancreas during Stage D, is discussed. The utilization of glycogen by the epidermis during Stages A and B, periods of inanition, is also discussed.

5. During Stages A and B alkaline phosphatase is heavily concentrated in the distal ends of the outer epidermal cells. It is observed in the integument in the region of the proximal portions of the pore canals, even before calcification begins. This is a region of high activity as deposition and hardening of the post-exuvial layers occurs. The enzyme is likewise found in the reserve cells of the subepidermal cells. In the hepatopancreas, the most marked localization of the enzyme is seen around the innumerable calcospherites in the absorption cells and in the striated border of these cells. The reserve cells of the hepatopancreas likewise show the presence of the enzyme. Functions of phosphatase in these sites are suggested.

6. Calcification begins the second day following molt and occurs thereafter simultaneously with, or immediately accompanying, the elaboration of layers of the principal zone. Calcification of the pre-exuvial layers, formed before molt, is a process accomplished long after their formation but during the same period at which the post-exuvial layers, formed after molt, are being calcified.

Calcium, heavily concentrated in the distal region of the epidermal cells, is extruded from this tissue in two distinct ways. As the post-exuvial layers are deposited, masses of calcium granules are simultaneously extruded, thus forming narrow bands paralleling the epidermis. In the pre-exuvial layers, on the other hand, calcium granules are observed in uniform vertical rows which emanate from the epidermis. These vertical rows of calcium granules correspond in location to the pore canals. Since calcification of the pre-exuvial layers occurs after their formation calcium must be transported and deposited some distance from the newly forming post-exuvial layers. This transport occurs through the pore canals, protoplasmic extensions of the epidermis, thus enabling this tissue to act at these distant sites.

7. While calcification of the integument occurs, the reserve cells in the subepidermal tissues undergo what appear to be cyclic peaks in calcium storing alternating with cyclic release to the epidermis. The reserve cells in this capacity could serve as reservoirs for providing additional calcium during periods of concentration by the epidermis. Furthermore, these interesting reserve cells, during the early postmolt period (Stages A and B), undergo at daily intervals, cyclic peaks and declines in size and abundance, changes in structural appearance, and staining properties and the storing of reserves other than calcium. The mucopolysaccharide material, either muco- or glycoprotein, in the reserve cells disappears near the latter part of Stage B. This indicates a decrease in the concentration of the material and suggests that the mucopolysaccharide stored by the reserve cells represents reserve material for the construction of the new skeleton.

The reserve cells of the hepatopancreas, like those of the integumental tissues, undergo during the early postmolt period (Stages A and B) cyclic peaks and declines in number, size and the storing and apparent release of reserves. They similarly show the presence of phosphatase, mucopolysaccharide, calcium and much lipid.

8. As calcification of the integument occurs there is a progressive decrease in number of calcospherites—spherules of calcium phosphate—present in the absorption cells of the hepatopancreas. These calcospherites, abundant preceding molt and on the first day following molt, progressively decrease in number as calcification of the new integument occurs and by the seventh day (end of Stage B) hardly a single calcospherite can be detected. The calcospherites, premolt storage depots of reserve phosphate from the old skeleton, probably represent a major source from which phosphate can be mobilized for hardening of the new skeleton during Stage B, a time at which the animals do not feed.

9. Development of the inner integument of the branchiostegites is completed in *Panulirus* during a period of three days preceding molt and two days following molt. Calcification of the inner integument, as in the pre-exuvial layers of the outer integument, occurs after its formation via the pore canals, and portions of this integument are completely calcified by the seventh day following molt (end of Stage B).

10. Calcium carbonate, the principal salt of the spiny lobster skeleton, constitutes approximately 42% of the total mineral deposited and is precipitated in the organic matrix as calcite, rather than aragonite or amorphous calcium carbonate.

11. The roles of carbonic anhydrase and citric acid in the calcification of the integument of Crustacea are discussed.

12. Continued accretionary growth of the skeleton and the hardening of it by calcification are two major tasks confronting the crustacean from Stage A to late Stage C. Although the epidermis takes the lead in the performance of these duties, the importance of the other tissues, namely the hepatopancreatic and sub-epidermal tissues, should not be under-estimated.

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DEVELOPMENTAL MODIFICATIONS IN THE SAND DOLLAR CAUSED BY COBALTOUS CHLORIDE IN COMBINATION WITH SODIUM SELENITE AND ZINC CHLORIDE[1]

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Studies on the effects of chemical agents on early developmental patterns in echinoderms have shown that similar modifications may often be caused by entirely different agents. Some groups of totally different substances will cause entodermization of the young embryo while others will produce the opposite effect (ectodermization) if concentrations and exposure periods are carefully controlled. Some will increase the area of the ventral field while others will decrease it. In recent studies (Rulon, 1952, 1953, 1955, 1956) it has been found that compounds such as sodium selenite, nickelous chloride, zinc chloride, and cobaltous chloride will all cause the loss of bilaterality and a polar elongation of the larva. Such larvae differentiate with reference to the new pattern and are very similar to each other, irrespective of the compound used.

When two unlike substances produce similar effects certain questions may be asked: (1) Do these agents affect identical reaction systems in the morphogenetic process? Or (2) are *different* loci in the reaction complex affected in such a way that the final results are the same? A definite answer to either question is difficult to make but it is believed that at least a partial answer may be suggested by the use of combinations of the different agents.

In our experiments it has been customary to submit the eggs and early embryos to a wide range of concentrations when testing the effects of any particular agent. This range usually extends from a concentration that is lethal in a few hours to one that has little or no effect. The range is commonly set up so that the succeeding steps are each one-half the concentration of the preceding (*i.e.*, $M/100$, $M/200$, $M/400$, $M/800$, $M/1{,}600$, $M/3{,}200$ . . .). When newly fertilized eggs from the same lot are distributed throughout the various concentrations, interesting comparisons may often be made. The stronger solutions commonly give proportionate inhibition—that is, all structures are grossly inhibited. With decreased concentrations the inhibition may be disproportionate (differential) in that certain processes or structures are strongly affected while others are affected slightly if at all. The differential inhibition of one structure often provides for the physiological release of another to the extent that the structure not inhibited increases in size beyond the normal.

The present work deals chiefly with combinations of agents. Since previous investigations have shown that cobaltous chloride, sodium selenite, and zinc chloride all cause the development of sand dollar embryos that are quite similar, it seemed important to determine if these agents could replace one another in inhibitory solutions. Would the effects of these different agents be antagonistic, additive, or possibly synergistic?

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Material and Methods

This work was conducted at the Hopkins Marine Station, Pacific Grove, California, during the summers of 1954–55. The adult sand dollars (*Dendraster excentricus*) were dredged from Monterey Bay and maintained in the laboratory in running sea water. Ovaries were exposed by removing the oral surfaces of the animals. The bright red eggs, exuding in droplets, were washed into finger bowls. After several washings in sea water the ova were fertilized by the addition of a few drops of sperm suspension. Only ova that were over 95 per cent fertilizable were used. All test solutions were made up in sea water and the controls and tests were always from the same batch of eggs. All eggs developed under uncrowded conditions in finger bowls, out of direct sunlight and under the moist conditions of the aquarium room where the temperature varied by no more than one degree from 18 degrees C. and smoking was not permitted.

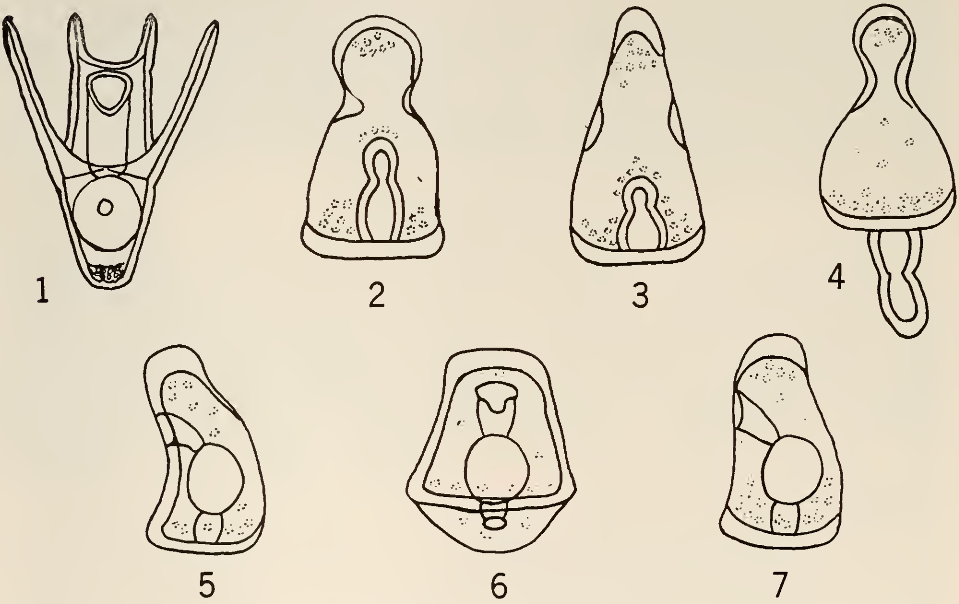
Experimental

1. *Continuous exposure of newly fertilized eggs to single and combination solutions of cobaltous chloride and sodium selenite.* It would be repetitious to report here all of the modifications caused by solutions of only cobalt and selenium (see Rulon, 1952, 1956). The object of this paper is to make comparisons between the effects of solutions containing the ions singly and in combinations at critical concentrations of the range. Accordingly, only the effects of the following solutions will be discussed although additional data have been obtained from various other concentrations and combinations:

Solution 1—Sea water control
2—*M*/800 cobaltous chloride
3—*M*/1,600 cobaltous chloride
4—*M*/800 sodium selenite
5—*M*/1,600 sodium selenite
6—*M*/1,600 cobaltous chloride—*M*/1,600 sodium selenite (50 cc. of *M*/800 $CoCl_2 \cdot 6H_2O$ plus 50 cc. of *M*/800 Na_2SeO_3)
7—*M*/3,200 cobaltous chloride—*M*/3,200 sodium selenite (50 cc. of *M*/1,600 $CoCl_2 \cdot 6H_2O$ plus 50 cc. of *M*/1,600 Na_2SeO_3)

After 48 hours almost all (98 per cent) in solution 1 (control) had developed into normal free-swimming bilateral plutei with well-differentiated oral and anal arms and with full skeletal development (Fig. 1). In solution 2 all were radial in symmetry and almost all (95 per cent) showed polar elongation (Figs. 2–4). Most showed differentiation of an apical lobe (Figs. 2, 4). All had thickened basal regions and commonly a basal circle of cilia was to be seen. Exogastrulation was present in 20–30 per cent and skeletal development was inhibited in all. All were slow-moving bottom forms. In solution 3, which was one-half the strength in cobaltous chloride of solution 2, less than 10 per cent resembled the radial elongated forms of solution 2. Instead, approximately 90 per cent were bilateral, free-swimming larvae (as Figs. 5–7). The majority of these larvae showed well-differentiated entera and stomodaea but no differentiation of skeleton or arms. There were some indications of polar elongation but they were not nearly so pronounced as in the larvae that had developed in solution 2. Approximately 20 per cent showed exogastrulation.

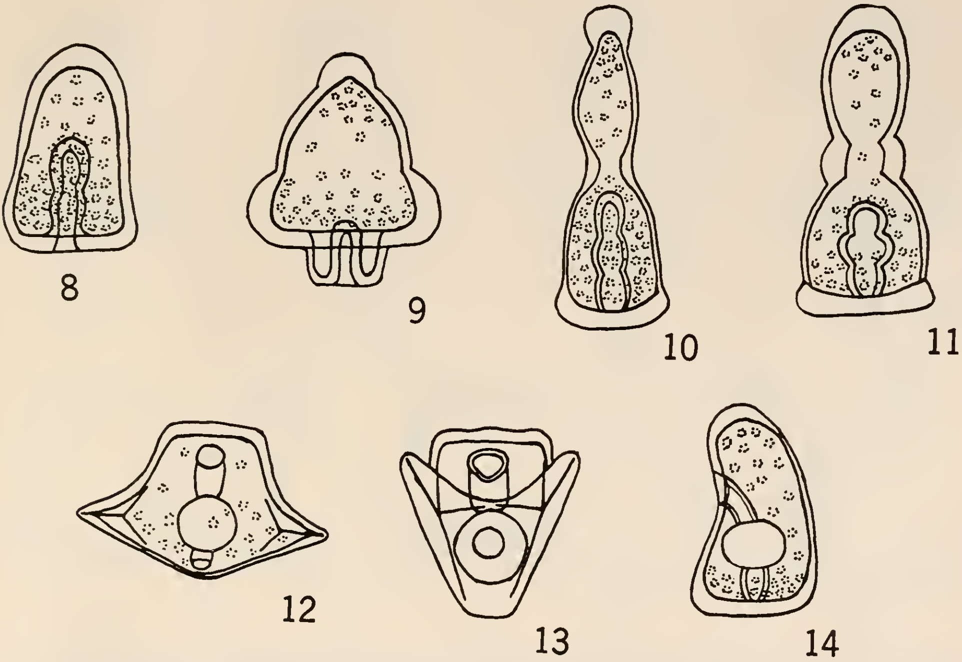
After 48 hours in solution 4 almost all (95 per cent) were radial forms with very little movement (Figs. 8–11). Over 75 per cent showed considerable polar elongation (Figs. 10–11) with differentiation of apical and basal lobes. No skeleton differentiated and many had basal ciliated bands. Only a few exogastrulae appeared. Approximately 75 per cent of the eggs developing in solution 5 resulted in bilateral plutei with short anal arms containing skeleton and at increased angles (Figs. 12–13). Oral lobes were broad but poorly differentiated. The remainder of the larvae in solution 5 graded from slightly bilateral forms without skeleton (Fig. 14) into the polar elongated radial larvae of higher concentrations (Figs. 10–11). Only an occasional exogastrula was seen.



FIGURES 1–7. Figure 1, normal 48-hour larva. Figures 2–4, 48-hour larvae that have been exposed continuously to $M/800$ cobaltous chloride. Figures 5–7, 48-hour larvae exposed continuously to $M/1{,}600$ cobaltous chloride.

Almost 100 per cent of the eggs developing in solution 6 became radial or near radial larvae (Figs. 15–18). Of these, over 50 per cent showed polar elongation with oral and basal lobes (Figs. 15–17). Approximately 10 per cent were exogastrulae. In solution 7, there was only an occasional elongated radial form while approximately 90 per cent were bilateral free-swimming larvae with ventral ciliated bands but no skeleton or arms (Figs. 19–21). Approximately 10 per cent were exogastrulae but the remainder had well-differentiated entera with stomodaea.

From these experiments it was shown that when the eggs of *D. excentricus* were exposed to a combined solution of cobalt and selenite ($M/1{,}600$ $CoCl_2$–$M/1{,}600$ Na_2SeO_3) they differentiated according to a pattern of radial symmetry very much as if they had been exposed to single solutions of double strength of either agent. That is, while $M/1{,}600$ cobalt alone caused 10 per cent radial and $M/1{,}600$ selenite alone caused 25 per cent radial, together they caused 100 per cent radial (approximately 100 per cent radial forms are caused by $M/800$ of either agent). In the case of polar elongation the combined solution gave a considerably higher percentage than did the single solutions ($M/1{,}600$ $CoCl_2$ or $M/1{,}600$ Na_2SeO_3) but not as high as single solutions of double strength. Exogastrulation was highest in the cobalt, next in the combination, and least in the selenite solutions. The data have demonstrated that the actions of these two different agents are additive in most respects in affecting developmental pattern but that there are also some effects caused by one agent but not (at least to an appreciable degree) by the other at the concentration used.

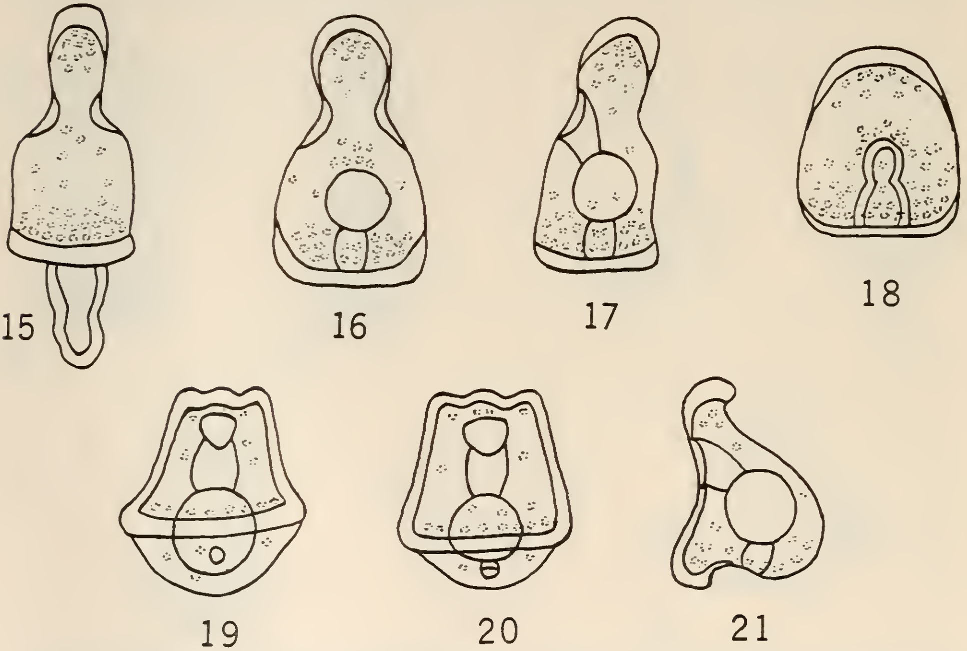


FIGURES 8–14. Figures 8–11, 48-hour larvae that have been exposed continuously to $M/800$ sodium selenite. Figures 12–14, 48-hour larvae that have been exposed continuously to $M/1{,}600$ sodium selenite.

2. *Continuous exposure of newly fertilized eggs to single and combination solutions of cobaltous chloride and zinc chloride.* As in the preceding experiments, eggs from the same lot were placed in wide ranges (single and combination) of concentrations. Since the single effects have previously been reported (Rulon, 1955, 1956) only the effects of selected solutions on development shall be reported here. The solutions were as follows:

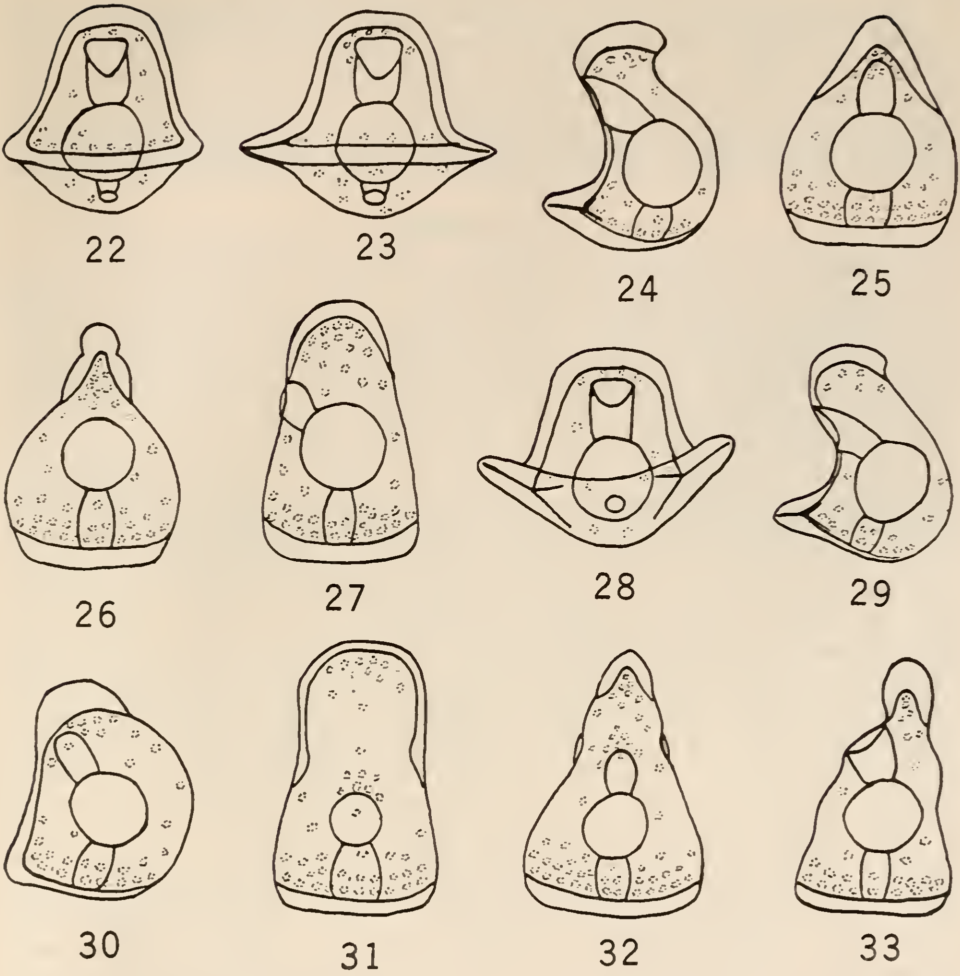
Solution 8—Sea water control
9—$M/3{,}200$ cobaltous chloride
10—$M/80{,}000$ zinc chloride
11—$M/160{,}000$ zinc chloride
12—$M/3{,}200$ cobaltous chloride—$M/160{,}000$ zinc chloride (50 cc. of $M/1{,}600$ $CoCl_2\cdot 6H_2O$ plus 50 cc. of $M/80{,}000$ $ZnCl_2$)

After 48 hours the eggs developing in the sea water control (solution 8) were practically 100 per cent normal free-swimming bilateral plutei with good development of oral and anal arms. In solution 9 all were slightly flattened bilateral larvae which were either actively swimming or moving about near the bottom of the culture (Figs. 22–24). Approximately 20 per cent had short anal arms with skeletal spicules and 10–20 per cent had undergone exogastrulation. While these larvae were definitely in advance of those treated with cobalt solutions of twice the strength (solution 3), they still showed markedly the effects of the agent.



FIGURES 15–21. Figures 15–18, 48-hour larvae that have been exposed continuously to a combination solution ($M/1{,}600$ cobaltous chloride-$M/1{,}600$ sodium selenite). Figures 19–21, 48-hour larvae that have been exposed continuously to a combination solution ($M/3{,}200$ cobaltous chloride-$M/3{,}200$ sodium selenite).

In solution 10 all of the larvae (Figs. 25–27) were slow-moving and radial with a large differentiated gut which commonly extended to the apical end (Fig. 25) although there were approximately 10 per cent which showed exogastrulation. These larvae had no skeleton and there was an excess of internal cells. Most showed apical thickenings or extensions although the polar elongation fell short of



Figures 22–33. Figures 22–24, 48-hour larvae that have been exposed continuously to *M*/3,200 cobaltous chloride. Figures 25–27, 48-hour larvae that have been exposed continuously to *M*/80,000 zinc chloride. Figures 28–30, 48-hour larvae that have been exposed continuously to *M*/160,000 zinc chloride. Figures 31–33, 48-hour larvae that have been exposed continuously to a combination solution (*M*/3,200 cobaltous chloride-*M*/160,000 zinc chloride).

that noted in certain effective solutions of cobalt and selenium. In solution 11 (which was one-half the concentration of zinc chloride as solution 10) over 90 per cent of the larvae were bilateral (Figs. 28–30) and over 50 per cent of these had short anal arms with skeleton. The remaining 10 per cent approached radial symmetry and there was approximately 5 per cent exogastrulation.

In solution 12 (combination solution) over 90 per cent were elongated radial forms with neither skeleton nor arms (Figs. 31–33). Slight bilaterality was evidenced in the remaining 10 per cent. Most had well-developed entera but 20–30 per cent had undergone exogastrulation.

These experiments show that zinc chloride causes modifications similar to those caused by cobaltous chloride but in concentrations that are 1/100th those of the latter. The addition of $M/3{,}200$ cobalt to $M/160{,}000$ zinc gives an effect which is approximately that of a single solution of zinc of twice the concentration ($M/80{,}000$) but not that of a single solution of cobalt of twice the strength ($M/1{,}600$). Instead the effect is that of a cobalt solution of four times the concentration ($M/800$).

DISCUSSION

Previous work by the author has shown that several substances are effective in causing polar elongation and differentiation around a radial symmetry in the developing sand dollar embryo. The present data show that cobalt and selenite at the same concentrations will produce such modifications. They also show that the effects of these two different agents were additive in causing radial symmetry and almost additive in polar elongation and exogastrulation.

Other work has indicated that the radial symmetry described here is the result of a process of ventralization (see Rulon, 1949) rather than a direct inhibition of the factors which give bilaterality to the normal embryo. It has been shown (see Child, 1941) that the ventral side of the early blastula has greater indophenol oxidase (cytochrome oxidase) activity than the dorsal side, even though visible morphological differences are not apparent. Neither cobalt nor selenite appears to inhibit the activity of ventral as much as dorsal regions. The ventral area therefore spreads until it encircles the entire embryo. It is suggested that the similar effects of cobalt and selenite may be related to their known inhibitory action of thiol groups (see Rulon, 1955) and that enzymes important in symmetry relationships bear active sulfhydryl radicals. It is further suggested that these enzymes are more dorsally located at the stage of development preceding visible bilaterality. Cytochrome oxidase, whose greater activity at the ventral side has been proven, does not possess an active sulfhydryl radical (Sumner and Somers, 1953, p. 9).

Polar elongation seems to have certain factors in common with ventralization. Child (1941) also showed a polar gradient of cytochrome oxidase activity in these eggs with the highest activity at the animal pole. If it is assumed that cobalt and selenite are inhibiting activities other than cytochrome oxidase and more basally located, then it would follow that the apical end would grow and become extended at the expense of basal regions.

Zinc was highly effective in causing ventralization and polar elongation, and at a concentration far below that of the other two agents. In other words, it was more effective in inactivating, or partially inactivating, certain of the factors or processes concerned with bilaterality and polarity. It may not be unreasonable to suggest that the affinity of the thiol groups of certain enzymes for this ion may be much greater than for cobalt or selenite ions. However, the picture may be more complicated, as shown by the fact that a solution of zinc ($M/160{,}000$) which is effective in causing 10 per cent radial forms will, when administered along with $M/3{,}200$ cobalt, which causes no radial forms, cause 90 per cent radials. This would seem to suggest a synergistic action although the complexity of the phenomenon does not lend itself to an easy interpretation.

Cobalt was much more effective in the concentration used than was selenite in causing exogastrulation and skeletal inhibition. This may be because the differentiation of gut and skeleton is of a more specific nature and therefore subject to more specific influences than is either ventralization or polar elongation.

SUMMARY

1. Newly fertilized eggs of *D. excentricus* were allowed to develop in single and combined sea water solutions of cobaltous chloride, sodium selenite, and zinc chloride.

2. Combination solutions of cobalt and selenite were additive in causing the development of radial larvae and almost additive in causing polar elongation of the larvae.

3. In solutions of equivalent strength the development of exogastrulae was highest in cobalt, next in the combination, and least in the selenite solutions.

4. Solutions of zinc caused effects that were similar to those of cobalt but in concentrations that were 1/100th the latter.

5. Combination solutions of cobalt and zinc gave effects that indicated synergistic action.

6. It is suggested that the effects of cobalt, selenite, and zinc are through their reaction with the thiol groups of certain enzymes and that the greater effect of zinc is because of a greater affinity for such groupings.

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NOTES ON THE LIFE-CYCLE OF AZYGIA ACUMINATA GOLDBERGER, 1911 (AZYGIIDAE-TREMATODA)

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Studies of trematodes belonging to the genus *Azygia* Looss, 1899 from North American fresh-water fish, date from the work of Leidy (1851). He described *Distoma longum* from the stomach of *Esox ester* LeSueur, 1818 from near Cleveland, Ohio. Since that time many workers have added to knowledge of the North American species belonging to this genus. Manter (1926) gave a systematic review of the family *Azygiidae* and stated (p. 57) that "Azygia is the only genus of the family showing taxonomic confusion in its species." He further pointed out that these forms are all very muscular and highly contractile, which not only alters the general shape of the worm but also changes the relative position of such structures as the acetabulum and the reproductive organs. He also mentioned that size of the eggs and distribution of the vitellaria vary considerably within a species and cannot be relied on as taxonomic characters. Manter recognized three valid North American species: *A. acuminata* Goldberger, 1911, *A. angusticauda* (Stafford, 1904) and *A. longa* (Leidy, 1851).

Van Cleave and Mueller (1934) endorsed the action of Manter in reducing the number of species in North America and felt that *A. acuminata* should also be reduced to synonymy with *A. longa*.

Stunkard (1956) gave a chronological account of the genus *Azygia* and noted (p. 266) "—discordant observations and divergent opinions," concerning the proposed specific and generic names for members of the genus. This thorough account need not be repeated here. Stunkard recognized *A. sebago* as a distinct species and suggested that the European *A. lucii* may also be present in North America and that it possibly is distinct from *A. longa*.

None of the species have been studied to determine the extent of variation that normally occurs as the result of early development in a wide variety of paratenic hosts (small fishes and planarians) and further development in varied definitive hosts (large fishes). Until such studies are undertaken, the taxonomic picture of the group will remain confused.

It is evident, in reviewing the literature, that species proposed by various North American authors and since placed in synonymy may indeed be valid species. In such worms, which are very muscular and highly contractile, size and shape can not be relied on as taxonomic characters except in a very general way. The fact that these worms become sexually mature while relatively small and continue to grow throughout life further complicates the taxonomic picture. *A. angusticauda* is the only North American species which can be readily distinguished from the other species described from this continent. It can be separated because of the more posterior position of the acetabulum and the presence of the gonads in the posterior one-sixth of the body (usually within the posterior one-seventh to one-eighth of the body). The rest of the species comprise a complex composed of *A. longa, A. sebago, A. lucii* and *A. acuminata.* This complex may perhaps include as yet unrecognized species, or species which at the present time are represented as identical to *A. longa.*

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The descriptions of the cercaria of *A. longa* by Sillman (1953a, 1953b), of the cercaria of *A. sebago* by Stunkard (1956), and the description of the cercaria of *A. acuminata* in the present report complete the life-histories of all of the recognized species of *Azygia* in North America with the possible exception of *A. lucii* and *A. angusticauda.* Dickerman (1937) named *Cercaria angusticauda* as a new species, but did not describe the larva.

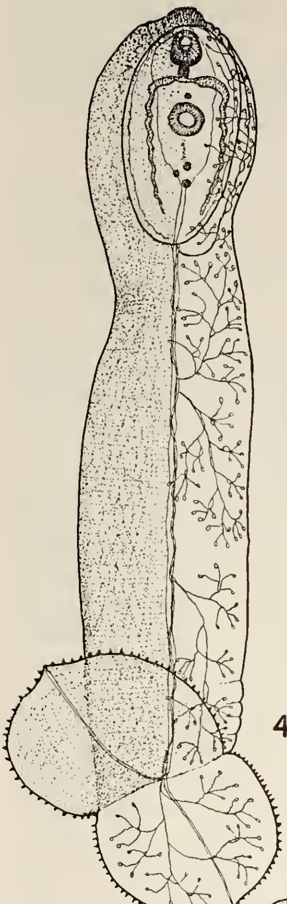
Moreover, in discussing the cystocercous cercariae of North America Horsfall (1934) pointed out that *C. wrighti* Ward, 1916, *C. anchoroides* Ward, 1916, and *C. brookoveri* Faust, 1918 appear to be typically azygiid in morphology and should logically develop into adult azygiids. One of these cercariae may develop into *A. lucii,* but if so it is morphologically distinct from the European larva.

Szidat (1932) showed that *Cercaria mirabilis* Braun is the larva of *A. lucii* in Europe. Sillman (1953a) described the larva of *A. longa* from the snail, *Amnicola limosa,* on the basis of experimental and limited natural infections. Sillman (1953b) added additional information concerning the life-cycle of *A. longa* which was reported from both the mud pickerel, *Esox vermiculatus* and *Amia calva* in the vicinity of Ann Arbor, Michigan. Sillman also assigned worms from the bowfin, *Amia calva,* to the suppressed species, *A. acuminata.* He was unable to experimentally obtain infections of *Amnicola* from eggs of *A. acuminata.*

Stunkard (1950) identified larval distomes from the pharyngeal pockets of planarians, *Dugesia tigrinum,* as immature azygiids. Planarians serve as paratenic hosts since further development of the distomes does not occur in this host. Stunkard (1956) reported on the life-cycle of *Azygia sebago* and experimentally showed that larval stages develop in *Amnicola limosa;* further that the cystocercous cercaria is distinct from that reported by Sillman (1953a, 1953b) from the same snail host. Stunkard also reported that these cercariae were ingested by small fish (guppies and small blue gill sunfish, *Lepomis macrochirus*) and also were obtained experimentally and naturally from the pharyngeal pockets of planarians, *Dugesia tigrinum.* He stated (p. 265), "How the larvae reach the pharyngeal pockets is not clear." On the basis of known feeding habits of planarians, he correctly suggested the manner in which the distomes enter the pharyngeal pockets but did not observe it.

In preliminary observations during the present study, cystocercous cercariae belonging to both *A. sebago* from *Amnicola limosa* and *A. acuminata* from *Campeloma decisum* (Say) were placed in finger bowls with *Dugesia tigrinum.* The planarians reacted to close proximity of both species of larvae by raising the anterior end of the body and, when the swimming cercaria came in contact with the under surface of the planarian, actively enclosing the cercaria against the glass container. The pharynx was then extruded and the cercaria was sucked into the protruded pharynx. Usually the tail entered first, always so in cercaria of *A. acuminata.* The distome portion of *A. sebago* was sucked into the pharynx where it was gradually divested of the tail and the distome became active and eventually

PLATE I



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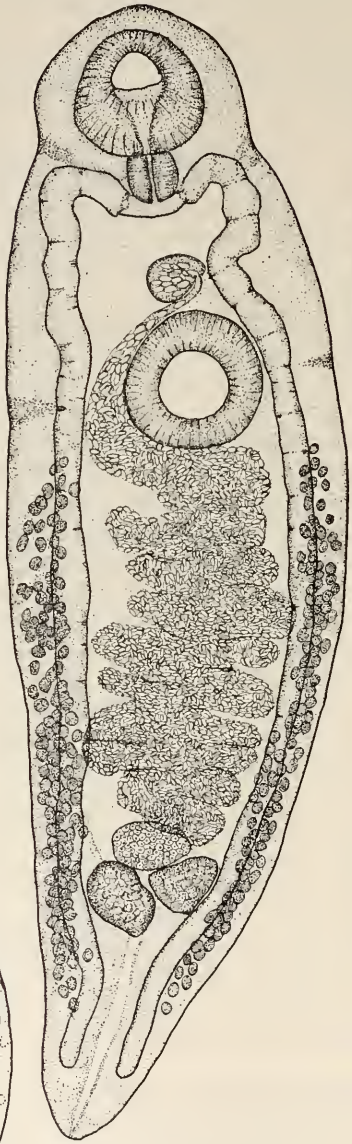

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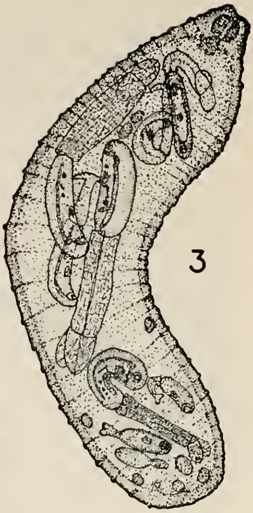

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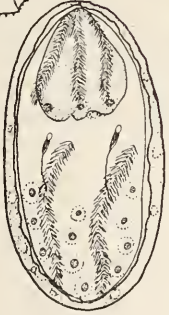

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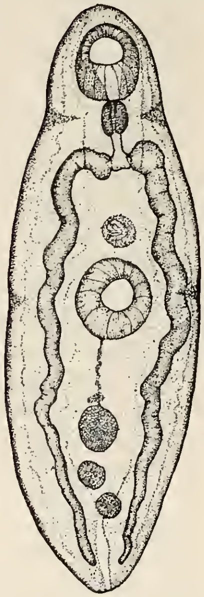

5

FIGURE 1. *Azygia acuminata,* adult (5.1 mm. in length), ventral view, collected from *Ameiurus nebulosus.*

FIGURE 2. *Azygia acuminata,* miracidium in egg (0.064 mm. in length), from sketches made of living larvae.

FIGURE 3. *Azygia acuminata,* redia (2.52 mm. in length), showing recognizable pharynx, annulations of the body and developing cercariae.

crawled out of the pharynx into the pharyngeal pocket. Up to six *A. sebago* larval distomes were seen to thus enter a single planarian. The larvae of *A. acuminata* were also enfolded by the planarians. The tail was sucked in but the larger size of the distome prevented both its entrance into the pharynx as well as into the aperture to the pharyngeal pocket. Occasionally a distome would be sucked part way into the pharynx and would remain so for several hours before finally becoming detached. Observations indicated that *D. tigrinum* could and probably does serve as a normal paratenic host for *A. sebago* but not for *A. acuminata.*

The cercaria of *A. acuminata* was found in *Campeloma decisum* obtained from the Santuit River, a small stream near the settlement of Santuit, Cape Cod, Mass. Small fishes, sticklebacks, *Eucalia inconstans* (Kirtland), *Fundulus heteroclitus,* the trout, *Salvelinus fontinalis* (Mitchill), and small eels, *Anguilla rostrata* LeSueur were obtained from streams known to be free of azygiid infections and were utilized as paratenic hosts. Specimens of each lot were examined and found to be free of azygiids and the remainder used, after infection, in feeding the definitive host fishes. These were found to be bull-heads, *Ameiurus nebulosus* LeSueur; blue gill sunfish, *Lepomis macrochirus* Raf.; chain pickerel, *Esox niger* LeSueur; and yellow perch, *Perca flavescens* (Mitchill). Yellow perch do not occur in this stream. These hosts were collected from a small pond known to be free of azygiids and were experimentally infected.

Large numbers of snails, *Campeloma decisum,* were kept in shallow, well aerated aquaria. In some instances liberated cercariae were pipetted from the aquarium and fed to small fish, and in other cases small fish were kept in the same aquarium. Heavy infections resulted in both cases since the swimming cercariae were readily ingested by the fish.

The young distomes differed in their relationship to the different species of small fish. Young trout and *Fundulus heteroclitus* retained an infection for only six to seven days. During this time the worms became less active and were found further and further posterior in the intestine of the fish. Sticklebacks and eels retained most of their infection in the stomach region. Worms removed from the latter species of fish were more active and their caeca were crowded with ingested material. Sticklebacks retained up to ten worms in the anterior portion of the intestine for as long as two weeks, whereas eels were found to have the stomach crowded with as many as twenty-four distomes.

Small fish were ingested by the larger fish used in the feeding experiments. The pectoral and dorsal spines of the sticklebacks were clipped before placing them in the aquarium with the definitive hosts.

Stages in the Life-Cycle

Adult (Fig. 1)

The original description of *A. acuminata* by Goldberger (1911) emphasized taxonomically-unimportant points such as zigzag caeca, rounded cephalic and

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Figure 4. *Azygia acuminata* cercaria, naturally emerged, from sketches of living specimen (4.20 mm. in length). Flame cell pattern of the tail, transposed from sketches made from other cercariae, shown on right side of tail. Detail of musculature shown on the left, omitted on the right side of the tail.

Figure 5. *Azygia acuminata,* juvenile worm (1.18 mm. in length), experimental infection, from stomach of small eel, *Anguilla rostrata.*

pointed caudal ends, uninterrupted vitellaria and constricted neck region in his specific diagnosis.

Manter (1926) studied Goldberger's material, also material identified by A. R. Cooper, and specimens from H. B. Ward's collection. He suggested that certain similarities in the material showed *A. acuminata* to be a valid species. Manter stated (p. 61), "The most distinguishing specific characters were found to be: relatively wide body, anterior extent of the vitellaria, egg size, and poorly developed condition of the internal parenchyma muscles. It should be realized that the nature of all of these features is of somewhat precarious standing in this group. Probably no one of them, unless very marked, would justify recognition of a separate species. Only because of the general association of all of these characters can the forms be separated from the other common American species."

Van Cleave and Mueller (1934) regarded *A. acuminata* and *A. bulbosa* of Goldberger (1911) as synonyms, and since Manter had regarded *A. bulbosa* as a synonym of *A. longa,* they added *A. acuminata* to the long list of synonyms of *A. longa.* They pointed out that while the anterior extent of vitellaria was an important taxonomic character in the genus, the comparative width, constriction of the neck, and insignificant differences in egg size did not justify maintaining *A. acuminata* as a valid species. They stated that the less highly developed condition of the internal muscles was a dubious character. On the basis of the present study it appears evident that Van Cleave and Mueller were not justified in reducing *A. acuminata* to synonymy with *A. longa;* that *A. bulbosa* should be considered synonymous with *A. acuminata;* and the species should be maintained in the genus *Azygia.*

Emended description of the adult

Characters of the genus, body, 4–10 mm. long, width usually less than one-fifth of the length. Body characteristically slightly constricted anterior to the acetabulum. Position of constriction variable depending on state of contraction of the worm. Acetabulum one-fourth to one-third total length from anterior end of body. Genital pore and cirrus sac median in position immediately cephalad of acetabulum. Oral sucker 0.25–0.90 mm. in diameter, acetabulum slightly larger, 0.28–0.95 mm. in diameter. Gonads contiguous. Posterior testis larger than anterior testis. Vitellaria extra-caecal, extending from level of posterior margin of acetabulum to level midway between posterior testis and caudal end of body. Eggs consistently larger than in other described azygiid species, 0.64–0.69 × 0.30–0.34 mm.

Host: *Amia calva, Lepomis macrochirus,* and *Ameiurus nebulosus.*

Habitat: Stomach.

Localities: Illinois, Michigan, and Cape Cod, Massachusetts.

Miracidium

Miracidia representing all genera of the family Azygiidae have been described except for the genus *Leuceruthrus.* Azygiid miracidia lack cilia and are provided with bristle plates or plaques. The miracidium of *A. acuminata* is morphologically similar to the miracidium of *A. sebago* described by Stunkard (1956), to that of *Proterometra macrostoma* as reported by Hussey (1945), and to the earlier descriptions of that of *A. longa* by Schauinsland (1883) and Looss (1894).

Eggs must be ingested by the proper snail hosts before hatching will occur.

Attempts to hatch the eggs by placing them with material from the digestive tract of large *Campeloma decisum* were unsuccessful.

Miracidia were studied both alive in the egg (Fig. 2) and in stained sections of gravid worms. The miracidium almost completely fills the egg-shell. Radiating from the anterior end are five plaques bearing fine bristles arranged in chevron fashion with the apices anterior in position. These plaques extend posteriad about one-third of the length of the larva. A short distance from the posterior end of the miracidium, four other bristle plaques extend anteriad with a tendency to spiral, which may be due to movements of the larva.

Internally, occupying almost the anterior one-third of the miradicium, are four unicellular gland-like structures, the so-called primitive gut of earlier authors, which Stunkard considered probably secrete substances which aid in penetration. Wootton (1957) demonstrated that in *Allocreadium alloneotenicum* this group of glands does aid in penetration and that the penetration glands of earlier authors serve in forming the cuticle of the sporocyst. Attempts, in crushed snails, to observe the action of the anterior gland in the miracidium of *A. acuminata* were unsuccessful. It is possible since ciliary plates are lacking in azygiid miracidia that the miracidial covering serves also as the covering of the sporocyst, thus explaining the absence of cuticle-forming glands. Up to twelve germinal cells are visible in the posterior two-thirds of the miracidium. Paired flame cells lie near the middle of the body, one on each side, each with a duct leading caudad. The ducts could not be clearly traced to their pores.

Sporocyst

Young snails were removed directly from the uterus of an uninfected female *Campeloma decisum*. The young snails readily fed in a layer of clean sand. Eggs of *A. acuminata*, each containing an active miracidium, were added to the sand in which the snails were feeding. Snails were dissected at the end of one, two, and three weeks but no infection was found. It is probable that the snails must be larger before infection will occur.

Rediae

Infections with larvae of *A. acuminata* were present only in females of *Campeloma decisum*. Infections of male *Campeloma* were never observed. The redial stages were present, usually with unencysted metacercariae identified as *Leucochloridiomorpha constantiae* (Mueller), in the uterus of the snail. The loci of infection did not extend into the digestive gland, the usual site of infection for larval trematodes. Up to eighty rediae were dissected from the uterus of a single snail. These, plus almost as many metacercariae of *L. constantiae*, completely filled the uterus. Embryo snails, which were usually found in the uterus of uninfected females, were absent in infected snails. In a few instances partially empty embryo snail shells were found in the uterus, but it was evident that infection with larval *A. acuminata* adversely affected normal development of the young snails. The redial stages undoubtedly derived their nourishment from the developing embryo snails within the uterus. Young snails develop normally when associated only with metacercariae of *L. constantiae*. Thus, infection of the snail with larvae of

A. acuminata caused the degeneration of the young snails. Rediae (Fig. 3) when fixed averaged 2.15 × 0.71 mm.

The vermiform rediae are very active and are capable of extending to a length of over 5 mm. When contracted, the external body wall is formed into regularly spaced annular rings, giving it a wrinkled appearance. Even when fully extended, these rings persist as fine annular projections. The body wall is 0.043 mm. in thickness in sectioned material. The small pharynx is not easily observable in stained whole mounts, but is visible in sections as a rudimentary structure (0.11 mm. in diameter) with a well defined lumen. No recognizable birth pore can be seen, either in sectioned materials or in stained whole mounts or rediae. It is probable that cercariae escape through the pharynx.

Up to twelve recognizable cercariae are present in each redia, with seven to ten being the usual number. Other developing cercariae are present as germ-balls, and a maximum number of twelve developing cercariae present with six germ-balls was observed. The redial stage is very similar to that reported by Szidat (1932) for *A. lucii* and by Stunkard (1956) for *A. sebago,* varying only in size and in number of developing cercariae.

Cercariae

The development of the cercaria is typical of that reported for cystocercous larvae. The tail becomes evident in development when the larva reaches a length of only about 0.1 mm. When the larva reaches a total length of 0.5–0.6 mm. the furcal buds appear as oval projections. At this stage the suckers are recognizable, and the primordia of one testis and the cirrus sac are also visible as deeper staining areas. The tail increases more rapidly than the distome during further development. The largest cercaria observed within a redia as a stained whole mount measured 2.22 mm. in total length. The distome measured 0.74 × 0.37 mm.

Cercariae appear to undergo additional development in the uterus of the snail before escaping from the female genital pore. After emergence, they are quiescent for a brief period before actively swimming in a typical cystocercous fashion. Cercariae normally emerge between 12:00 P.M. and 4:00 A.M. Standard Time. Limited numbers escape during daylight hours. It appears, however, that the majority emerge during hours of darkness and are either ingested by small fish at this time or in the early hours of daylight. They live for only ten to twelve hours, becoming less active as they age. Cercariae while still within the uterus of the snail do not have the distome portion enclosed by the tail. Upon coming into contact with the water, the anterior tail bulb absorbs water, expands rapidly anteriorly thus enclosing the distome, as has been described for other cyctocercous cercariae.

Mature, normally liberated cercariae (Fig. 4) measure 3.21–4.69 mm. (averaged 4.22 mm.) in total length when infected snails are first brought into the laboratory. The size of the cercariae gradually decreases in snails kept in captivity, undoubtedly a result of deficient nutrition of the hosts. The tail stem is round in cross-section at the bulb-like anterior end enclosing the distome. This portion measures 0.69–0.96 mm. in diameter (average 0.78 mm.). Just posterior to the more or less rigid anterior bulb enclosing the distome, the tail decreases slightly to a diameter of 0.52–0.82 mm. (average 0.66 mm.). From the constricted neck-portion the tail gradually flattens and widens to a width of 0.62–1.11 mm. (average 0.84 mm.) and then tapers gradually to an average width of 0.79 mm. just anterior to the furcal branches.

The furci are broadly lobed structures, 1.11–1.28 mm. in length (average 1.21 mm.) and 0.89–1.09 mm. in width (average 0.94 mm.). Each furca has a terminal papilla on which the excretory pore opens and small regularly arranged scale-like marginal protuberances. The tail of the cercaria is colorless, slightly opaque and devoid of protuberances, spines and mammulations characteristic of *C. mirabilis* Braun, 1891, *C. macrostoma* Faust, 1918, *C. splendens* Szidat, 1932, *C. anchoroides* Ward, 1916, and *C. sebago* Stunkard, 1956. The cercaria of *A. acuminata* differs in size and in the proportionate size of the distome when compared with the tail length from other cystocercous cercariae which characteristically do not possess papillae. It differs from *A. hodgesiana* Smith, 1932 since the genital organs are not functional as they are in the latter; from *A. stephanocauda* Faust, 1921 in size and shape of the tail; from *C. wrighti* Ward, 1916 in size; and from *C. pekinensis* Faust, 1921 in proportionate size of the distome.

The cercaria of *A. acuminata* is most like *C. brookoveri* Faust, 1918 and *C. anchoroides* Ward, 1916, but is over twice as large. *C. brookoveri* was originally described from crushed *Campeloma* sp. and the free-swimming larva was rediscovered by Dickerman (1937) from the same snails. Unfortunately Dickerman did not further describe the species. *C. anchoroides* was collected only in plankton tows from Lake Erie. The size and obvious similarities in structure of the two forms, as well as geographic proximity of the type localities, caused Horsfall (1934) to think that they will prove to be synonymous when the life-cycles are known.

The enclosed distome measures 0.66–0.79 × 0.37–0.47 mm. in living material. It usually lies with the oral sucker at the anterior end of the tail-bulb. It is flattened and varies as to its orientation to the width of the tail, sometimes being at right angles and at others with its width the same as the width of the tail. The excretory system is continuous with that of the tail, extending down the tail as a common excretory canal bifurcating at the bases of the furci and opening at the small points of the furci.

The structure of the larva when forced from the tail-bulb is typically azygiid. The preacetabular region bears many papillae which decrease in size and number, and are absent behind the mid-acetabular region. Living specimens, flattened slightly under a cover glass, measure from 0.98–1.38 mm. (average 1.18 mm.) in length and from 0.37–0.54 mm. in width (average 0.48 mm.). The oral sucker varies from 0.22–0.25 mm. in length and from 0.20–0.23 mm. in width. It is subterminal, opening ventrally. The pharynx measures 0.090–0.098 mm. in length and 0.49–0.61 mm. in width. The acetabulum varies from 0.22–0.25 mm. in length and from 0.25–0.29 mm. in width. It is about midlength in the body. The digestive caeca are filled with opaque material and extend almost to the posterior end of the larva. The excretory bladder extends anteriorly to the region just posterior to the testes where it branches into two main collecting ducts. These extend median to the caeca, crossing laterad as the caeca turn mediad to join the pharynx. After crossing under the caeca, the ducts pass laterally and antero-laterally to the oral sucker, continuing almost to the anterior end of the body, but they do not join. Anterio-lateral to the oral sucker each duct doubles backward and extends posteriad, lateral to the caeca, giving off eleven branches.

The first branch is located lateral to the oral sucker, the second at the level of the pharynx, the third and fourth anterior to the acetabulum, the fifth at the anterior edge of the acetabulum, the sixth lateral to the acetabulum, the seventh and eighth are close together just behind the acetabulum, the ninth and tenth are about equally spaced in the intervening region, while the last branch continues to the posterior end of the body lateral to the excretory bladder. Each branch divides three times in a dichotomous fashion, thus forming two primary, four secondary, and eight tertiary branches. Each tertiary branch drains four flame cells. Thus the flame cell formula is 2 (11 × 32) or 704 flame cells.

The number and arrangement of the flame cells is in agreement with those reported by Looss (1894) for *Azygia terreticolla* (= *A. lucii*) and by Stunkard (1956) for *A. sebago*. While the numbers of branches and flame cells agree with these earlier descriptions, the positions of the branches are different in *A. acuminata* due to the relatively more posterior position of the acetabulum.

The excretory system of the tail is equally complex (Fig. 4). In addition to the common excretory canal extending down the center of the tail and bifurcating into each furca, two paired accessory canals paralleling the main canal were observed. One duct and its branches drained the right side of the tail and the right furca, and the other the left side and the left furca. Each duct collected from five branches but dichotomous bifurcation of the ducts was not as clearly evident as in the distome portion.

Each of the five branches, however, did drain from 32 flame cells, arranged in groups of fours. The first branch turned anteriad from just caudad of the distome, draining the enclosing bulb area, the second and third branches joined the collecting duct close together in about the middle of the tail, the second turning anteriad and draining that area, the third posteriad collecting from the third quarter of the tail. The fourth branch joined the collecting ducts about three-fourths the length of the tail and drained the final quarter of the tail. From the fourth branch, the duct extended into a furca draining from 32 flame cells. The formula for the tail is thus 2 (5 × 32) or 320 and the entire cercaria has a formula of 2 (16 × 32) or 1,024 flame cells.

The connection of the accessory ducts of the tail to the rest of the excretory system was not resolved. The dense protoplasm at the tip of the tail in immature cercariae freed from rediae and the congested area at the base of distomes enclosed in the tail of normally liberated cercariae made observations impossible. These ducts are 0.011 mm. in diameter compared to the common duct which is 0.055 mm. in diameter. Faust (1921) reported that in *C. pekinensis,* the tail had only 32 flame cells and connected to the excretory system of the distome as the eighth branch. He further reported only seven branches in the distome portion of *C. pekinensis.* The flame cell pattern of this form should be examined in the light of the observations of the cercariae of *A. lucii* Looss (1894), *A. sebago* Stunkard 1956, and the present observations of *A. acuminata,* since *C. pekinensis* would appear to also develop into an azygiid.

Young worms

Worms increased very little in size and did not undergo further development while in the stomach of small fishes. A young distome (Fig. 5) from the stomach of a small eel differed from one newly forced from the cercarial tail only in the size of the caeca. In worms taken from paratenic hosts, the caeca were enlarged with food particles. No measureable differences in worms from various hosts were found. Worms from the sticklebacks and young eels were more active and appeared healthier than worms from other small fish.

Manter's synopsis and key to the genus *Azygia* can be revised to include *A. sebago* and the cercaria of each species can be noted as follows:

KEY TO THE SPECIES OF AZYGIA FROM NORTH AMERICA

1 (2) Vitellaria not extending appreciably posterior to the last testis. Length 6–54 mm. (*C. mirabilis* Braun, 1891) .... *A. lucii* (Mueller)
2 (1) Vitellaria extending posteriad at least half the distance between posterior testis and end of body 3
3 (4) Acetabulum near middle of body, gonads in posterior one-sixth of body (*C. angusticauda* Dickerman, 1937) .... *A. angusticauda* (Stafford, 1904)
4 (3) Acetabulum within anterior one-third of body, gonads more anterior 5
5 (6) Body width usually one-fifth the total length, vitellaria extending posteriad from close behind acetabulum, internal parenchyma muscles relatively weak. Eggs 0.064–0.069 × 0.30–0.34 mm. (*Cercaria acuminata,* present paper) ..... *A. acuminata* Goldberger, 1911
6 (7) Body width proportionately less than one-fifth the length, vitellaria begin some distance posterior to acetabulum, internal parenchyma muscles strongly developed, eggs variable in size but smaller than *A. acuminata* 7
7 (8) Body length not over 15 mm., usually smaller, body robust in appearance (*Cercaria sebago* Stunkard, 1956) .... *A. sebago* Ward, 1910
8 (7) Body often extremely elongate, vitellaria beginning proportionally more posteriorly (*Cercaria longa* Sillman, 1953a) .... *A. longa* (Leidy, 1851).

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SUMMARY

1. Stages in the life-cycle of *Azygia acuminata* are described and figured. Cystocercous cercariae develop from rediae in the snail, *Campeloma decisum.* The cercaria is morphologically distinct from other described cystocercous cercariae. Rediae are similar to the same stage described for other members of the genus, but are unique since they develop in the uterus of female *Campeloma decisum.*

2. The excretory system of the cercaria is complex, showing a formula of 2 (11 × 32) or 704 flame cells for the distome portion and 2 (5 × 32) or 320 flame cells in the tail. The excretory formula of the cercariae is thus 2 (16 × 32) or 1,024 flame cells.

3. Attempts to experimentally infect small snails, taken from the uterus of a *Campeloma decisum,* by feeding them eggs of *A. acuminata* were not successful.

4. Various small fishes were utilized as paratenic hosts by the young distomes. Infections in sticklebacks, *Eucalia inconstans,* and small eels, *Anguilla rostrata,* resulted in more active and vigorous worms than did infections from other paratenic hosts.

5. The variation that normally occurs in members of the genus *Azygia* due to development in a wide variety of hosts is not known. Consequently diagnostic characters of mature worms can not be relied on to distinguish species. On the basis of this report the suppressed species *A. acuminata* is regarded as a valid species and should be retained in the genus *Azygia.*

6. *A. acuminata,* previously reported only from *Amia calva,* was found occur-

ring naturally in bullheads, *Ameiurus nebulosus,* blue gill sunfish, *Lepomis macrochirus,* and chain pickerel, *Esox niger,* from Santuit River, Barnstable County, Cape Cod, Massachussetts. Experimental infections were also obtained in these fishes and in the yellow perch, *Perca flavescens.*

7. A revised key for the genus *Azygia* is presented, listing the recognized species and the described cercarial stages.

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